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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Alimentar, realizada sob a orientação científica da Doutora Elisabete Verde Martins Coelho, Investigadora de Pós-Doutoramento do Departamento de Química da Universidade de Aveiro e da Doutora Isabel Henriques, Investigadora Auxiliar do CESAM – Centre for Environmental and Marine Studies, Departamento de Biologia da Universidade de Aveiro.

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Palavras-chave

Óleos essenciais, Subprodutos de segurelha, atividade antimicrobiana, subprodutos de cebola, fibra solúvel, análise de açúcares

Resumo

Na indústria avícola, os fitobióticos e os prebióticos têm sido investigados como alternativas naturais aos antibióticos promotores de crescimento (AGPs). O presente trabalho avaliou o potencial antimicrobiano dos subprodutos agroindustriais de *Satureja montana* e a presença de potenciais moléculas prebióticas em subprodutos de *Allium cepa* como possíveis ingredientes em alimentos compostos para frangos de aviário.

Óleos essenciais (EOs) foram obtidos a partir de caules de *Satureja montana* por hidrodestilação (≈120 min) com aquecimento por convecção, e por hidrodestilação assistida por micro-ondas (16 ± 1 min) a partir de caules re-hidratados e escorridos, obtendo-se rendimentos similares de 0,14 ± 0,07 e de 0,10 ± 0,01 mg/g, respetivamente. A análise por CG-MS revelou que os EOs extraídos por diferentes metodologias e de diferentes colheitas apresentaram composição química semelhante. Foram identificados 13 compostos nos EOs, dos quais 84,4% - 97,6% são monoterpenóides e 0,3 - 0,5% sequiterpenóides, sendo o carvacrol o composto maioritário (825 – 950 µg/mg). A atividade antimicrobiana dos óleos essenciais foi avaliada em *Escherichia coli* ATCC 25922, *Salmonella enterica* sv Anatum SF2 e *Staphylococcus aureus* ATCC 6538 utilizando um método de difusão em disco e um ensaio de microdiluição em meio de cultura líquido. Todos os microorganismos testados foram inibidos pelo óleo essencial de *Satureja montana*. As estirpes de bactérias Gram-negativas (*E. coli* 25922 e *Salmonella* SF2) foram muito menos suscetíveis do que as bactérias Gram-positivas (*S. aureus* 6538). A atividade do óleo essencial testado está relacionada com presença de uma elevada quantidade de carvacrol, obtendo-se valores de concentrações mínimas inibitórias (225 para *E. coli* 25922, 250 para *Salmonella* SF2 e 150 µg/mL para *S. aureus* 6538) muito similares aos valores reportados para o carvacrol nestas estirpes.

A partir dos subprodutos da cebola foram obtidos extratos com etanol:água (8:2) em ebulição e extratos aquosos a 80°C sequencialmente. Comparativamente foi efetuada uma extração com água superaquecida e com etanol:água (65:35) por micro-ondas a 120, 140 e 160°C. A composição dos açúcares e a análise das ligações glicosídicas mostraram a presença de polissacarídeos pécticos e glucose em ligação terminal, provavelmente derivada de compostos fenólicos glicosilados. Dentro dos oligossacarídeos detetados, todos os extratos apresentaram apenas dissacarídeos (nomeadamente sacarose e trealose) numa quantidade inferior a 7 µg/mg. No entanto, a presença de prebióticos, tais como a inulina e frutooligossacarídeos, foram diagnosticados pela ligação (1 → 2)-frutose nos subprodutos de cebola, em baixas quantidades.

A fibra dietética solúvel dos subprodutos de cebola tem potencial para ser explorada como prebiótico. Conclui-se que os subprodutos de *Satureja montana* possuem potencial antimicrobiano contra as principais bactérias patogénicas das aves, mostrando-se promissores como alternativa na substituição / redução dos antibióticos em alimentos compostos para aves.

Keywords

Essential oils, Winter savory byproducts, antimicrobial activity, onion byproducts, soluble fiber, sugar analyses

Abstract

Phytobiotics and prebiotics have been investigated as natural alternatives to antibiotic growth promoters (AGPs) in poultry industry.
The present work evaluated antimicrobial activity of agroindustrial *Satureja montana* byproducts and the presence of potential prebiotic molecules in *Allium cepa* byproducts as possible ingredients in broiler diets.
Essential oils (EOs) were obtained from *Satureja montana* branches by hydrodistillation (≈120 min) with convective heating, and by microwave assisted hydrodistillation (16±1 min) of rehydrated and drained branches, obtaining similar yields of 0.14±0.07 and 0.10±0.01 mg/g, respectively. GC-MS analysis revealed that the EOs obtained by different extraction methodologies and harvest had similar chemical composition. A total of 13 compounds were identified. Monoterpenoids account for 84.4 – 97.6% and sequiterpenoids 0.3 – 0.5%, being carvacrol the highest component (825 – 950 µg/mg). Antimicrobial activity of essential oils was individually evaluated against *Escherichia coli* ATCC 25922, *Salmonella enterica* sv Anatum SF2 and *Staphylococcus aureus* ATCC 6538 using an agar disc diffusion method and broth microdillution assay. All tested microorganisms were inhibited by EO obtained from byproducts of the *Satureja montana*. Gram-negative bacteria strains (*E. coli* 25922 and *Salmonella* SF2) were much less susceptible than Gram-positive bacteria (*S. aureus* 6538) to the tested EO. The activity of tested EO is related to the presence of high levels of carvacrol, obtaining minimal inhibitory concentration (225 for *E. coli* 25922, 250 for *Salmonella* SF2, and 150 µg/mL for *S. aureus* 6538) similar to carvacrol in same strains.
Onion byproducts extracts were obtained by sequential extraction with boiling ethanol:water (8:2) and water at 80°C. In comparison, microwave superheat water and ethanol:water (65:35) extracts were obtained at 120, 140, and 160°. Carbohydrate composition and glycosidic linkage analysis revealed that the presence of pectic polysaccharides and terminally-linked glucose probably from glycoconjugated phenolic compounds. Within the detected oligosaccharides, all extracts showed only disaccharides (namely sucrose and trehalose) in an amount of less than 7 µg / mg. However, prebiotics such as inulin and fructooligosaccharides could be diagnostic by (1→2)-linked fructose in the onion byproducts at low amounts.
The soluble fiber from onion byproducts have potential to be exploited as prebiotics. It is concluded that the *Satureja montana* byproducts have an antimicrobial potential against the main pathogenic bacteria of the birds becoming promising as an alternative in the substitution / reduction of the antibiotics in the feeds.

Index

List of Figures

List of Tables

Acronyms

1-PECSO - *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulphoxide

ACSOs - S-alk(en)yl-L-cysteine sulphoxides

AGP - Antibiotic growth promoters

AXOS - arabinoxylooligosaccharides

BMD - Bacitracin methylene disalicylate antibiotic

BWG - Body weight gain

DF - Dietary fiber

DM - Dried matter

DMSO - Dimethylsulphoxide

EOs -Essential oils

FCR -Feed conversion rate

FI - Feed intake

FFT - fructan: fructan fructosyltransferase

FOS – fructo-oligosaccharide

GC-MS - Gas chromatography couple to mass spectrometry

HD- Hydrodistillation

IDF – insoluble dietary fiber

MCSO - (+)-*S*-methyl-L-cysteine sulphoxide

MIC - Minimum inhibitor concentration

MW-HD - Microwave assisted hydrodistillation

MOS - mannooligosaccharide

PCSO - (+)-*S*-propyl-L-cysteine sulphoxide

PKM - Palm kernel meal

PMAAs - Partially methylated alditol acetates

POS- pectic oligosaccharides

SCFAs - Short-chain fatty acids

SDF – soluble dietary fiber

SST - sucrose: sucrose fructosyltransferase

OHWE- Overplus hydration water extract

XOS – Xylo-oligosaccharides

1. Theoretical framework and objective

Livestock is a high growing sector in the agricultural economy. Actually, it contribute 40% of the global value of agricultural output and support the livelihoods and food security of almost 1.3 billion people (1). Worldwide, in 2014 more than 100 million tons of chicken meat were produced, 40 million tons more than in 2000 (2). These levels of world production are sustained mainly by large productions, where food-animals are often housed with high animal densities, facilitating the introduction and spread of disease. Meat producers have the main objective of increasing the weight of the animals so that they are ready for slaughter even before they have reached physical maturity (3).

A wide range of antibiotics (natural, synthetic or semi-synthetic) are used worldwide within the poultry industry for three purposes: therapeutic use to treat sick animals; prophylactic use to prevent infection in animals; as growth promoters to improve feed utilization and production (4). These uses of antibiotics are dependent on the legislation in each continent and country, level of industrialization of animal production and characteristics of the market for food animal products. In developed nations, about 50-80% of all antibiotics are used in livestock consume (5). In countries that still allow the use of antibiotic growth promoters (AGP), such as the USA, more than half of the antibiotic given to animals are used for this purpose (5). The antibiotic growth promoters (AGP) are administered at a subtherapeutic dosage and increase growth rate as a result of improved gut health, resulting in better nutrient utilization and thus improving the productivity and economic returns to farmers (6). However, the use of dietary antibiotics have resulted in problems such as development of antibiotic resistant bacteria and occurrence of drug residues in the final products, which may trigger adverse reactions in consumers with hypersensitivity (4,6,7). AGP are generally not metabolized in the animal's body and therefore they are bioaccumulated in large concentrations in the tissues or alternatively are excreted in urine, feces and eggs, promoting the dissipation of antibiotic residues through the food chain or the environment (6). The development of microbial resistance occurs as a response of the bacteria to the wide use of antibiotics and their presence in the environment when certain mutations occur. Some mutations enable the bacteria to inactivate antibiotics or eliminate the cell target that the antibiotic attacks. These genetic elements can be transferred to other organisms, dissipating the resistance and putting human health and safety at risk (3,8).

In order to prevent the emergence of infectious diseases and antibiotic resistance, new international laws and cooperative efforts are needed. In European Union, all AGP were

legislatively withdrawn from poultry feeds beginning January 1, 2006 (17). It has become necessary to develop alternative substances and strategies for animal growth promotion and disease prevention. Within the farming practices and breeding programs, it has been proposed to reduce animal density and improve hygiene conditions in order to reduce exposure to pathogenic bacteria and viruses (7). In recent years several alternative substances have also been proposed so that the health and immune status of poultry are improved, including phytobiotics and prebiotics (9–11). Obtaining these substances can be achieved through food wastes, promoting a circular economy.

The food industry generates large volumes of wastes and byproducts. The exponential growth of the population and the growing demand for food combined with the current absence of effective waste management strategies leads to the accumulation of food wastes, consequently this possess serious ethical, economic and environmental issues (12). In Europe, each year about 90 million tons of food (or 180kg per person) are wasted and one third of the food produced is lost or wasted. It is estimated that 39% these losses occur in the food processing and manufacturing industry (13). Alternative practices to minimize and valorize waste are necessary to achieve more efficient resource use, thus promoting sustainability. In this sense it emerged the concept of industrial ecology that aim at "zero waste" society and economy where wastes are used as raw material for new products and applications within an industrial symbiosis. The first step for applying industrial symbiosis concept in the food processing sector is the identification, quantification and characterization of residues to try to understand which high-added value ingredients can be recovered from these and which techniques should be applied for their recovery (14).

The present work evaluated antimicrobial activity of agroindustrial winter savory byproducts and the presence of potential prebiotic molecules in onion byproducts as possible ingredients in broiler diets. Among the byproducts of the onion, the skin will be used for this study since this is the major waste produced during processing (15), and previous studies revealed high dietary fiber content in this part of the onion (16). The oligosaccharides and polysaccharides that constitute this fiber may potentially play a prebiotic role when present in the chicken colon, modeling the gut microbiota and promoting a general state of health (17). Among the byproducts of the savory, the branches after leaves removal were used, since they are generated in large quantities and are discarded, and still have no profitable application. From these branches essential oils (EOs) will be obtained since that EOs potentially reduce levels of pathogenic bacteria and may enhance immune status when inserted into poultry diets (18). Essential oils were obtained by two different methodologies (hydrodistillation and microwave assisted hydrodistillation) and onion byproducts extracts were obtained using

different temperatures and solvents. Afterwards, were performed the quantification and characterization of essential oils composition in terpenic compounds and from the onion byproducts extracts were also performed the quantification and characterization the composition in sugars. The obtained EO and fractions obtained were assayed to the antimicrobial and prebiotic effect, *in vitro*.

2. Agrofood byproducts as alternatives to antibiotics as health-promoting agents in poultry

2.1 Antibiotic growth promoters and their action mode

Antibiotic growth promoters (AGPs) revolutionized the intensive poultry production since the late 1940s. The low cost and the easy availability of antibiotics are factors that contributed to their wide use. In Europe, the most commonly used AGPs in aviaries were avoparcin, virginiamycin, bacitracin, spiramycin, tylosin, and virginiamycin (a streptogramin combination) (19,20).

AGPs improve growth rate and feed conversion efficiency but it is not totally clear how these compounds exert their beneficial action. It has been proposed that these compounds act in two ways: 1) AGP mediated growth through their antimicrobial activity on gut microbiota, especially by inhibiting pathogens and bacteria that cause growth depression, and 2) AGP directly interact with the complex intestinal ecosystem, especially with those innate immune cells mediating inflammatory response(21).

Their antimicrobial activity on gut microbiota has been supported by observations that showed that AGPs do not exert growth-promoting effects in germ-free animals. In fact, gut microbiota has significant effects on host nutrition, health, and growth performance and it is estimated that in pathogen-free environment the chickens growth is 15% faster than in conventional conditions (11). When AGPs are added to broiler feed shifts in the composition of the microbiota are noted because although the AGPs are used in subtherapeutic or sub-minimum inhibitor concentration (MIC) can cause growth-inhibition of certain bacterial species including pathogens (11,22). Consequently, there is a reduction in bacterial production of growth-depressing metabolites and in bacterial utilization of essential nutrients, an increase of vitamins and other growth factors synthesis, and an improvement of the nutrients absorption by reducing the thickness of the intestinal epithelium (22).

AGPs are capable of manipulating inflammatory innate immune response (22,23). It was proposed that AGPs can be accumulated in phagocytic cell (macrophages and polymorphonucleocytes) and inhibit one or more functions of these inflammatory cells, including chemotaxis, production of reactive oxygen species and proinflammatory cytokine production (23). Inflammatory symptoms, such as loss of appetite and muscle tissue catabolism, are attenuated, which reduces energy wasting and spares energy for animal grow (23).

The use of AGPs had productive advantages for the chicken industry, however, the emergence of resistance among pathogenic bacteria and parasites put human health in risk. This has led the European Union to legislatively withdraw all AGP from poultry feeds beginning January 1, 2006 (21). Hence, it is required the creation of alternatives to the use of AGPs that sustain intensive modern farming.

2.2 Characteristics of an AGP alternative

With the abolition of AGPs it has become urgent to look for alternatives that could replace antibiotics without causing loss of productivity or product quality. The world population is increasing and as a consequence a greater demand for meat and derivatives will be denoted (21). Scientists need to consider addressing these challenges and search sustainable alternatives for AGPs thus promoting food safety and environmental conservation (21). Yadav *et al*.(9) brought together a set of attributes that these alternatives to AGPs must have: "1. It should improve performance effectively; 2. It should have little therapeutic use in human or veterinary medicine; 3. It should not cause deleterious disturbances of the normal gut flora; 4. It should not be involved with transferable drug resistance ; 5. It should not be absorbed from the gut into edible tissue; 6. It should not cause cross-resistance to other antibiotics at actual use level; 7. It should not promote *Salmonella* shedding; 8. It should not be mutagenic or carcinogenic; 9. It should not give rise to environmental pollution; 10. It should be readily biodegradable; and 11. It should be non-toxic to the birds and its human handlers." In addition to this, products that are currently part of the human food chain should not be used as alternatives to AGPs in order to avoid food crises or higher food prices. Besides that, for the application of a given alternative, its inherent cost will be a preponderant factor in decision-making. Food byproducts are thus an option to consider since they may be economical and sustainable sources of AGPs, however, it has to be assessed whether the remaining requirements are met.

2.3 Agrofood byproducts as alternatives for Antibiotic Growth Promoters (AGP)

Several classes of alternatives have been proposed and tested in poultry production, including probiotics, prebiotics, symbiotics, organic acids, enzymes, phytogenics, metals, hyperimmune egg yolk antibodies, antimicrobial peptides and bacteriophages (24). These alternatives may partially compensate but do not completely replace the use of AGPs. Some of these classes of alternatives can be found in agrofood byproducts and a single byproduct may contain more than one class of these alternatives. The incorporation of a byproduct or it

fractions into animal diets can have repercussions on beneficial health and performance effects. Often it is not easy to establish a causal line between the presence of a particular compound and the beneficial effects generated since these effects result from a combination of interactions between the various components of the complex food matrix and the animal. Then a summary of the prebiotics and phytobiotics effects obtained after the incorporation of byproducts or fractions in the chickens´ feeds will be presented.

2.3.1 Prebiotics

Prebiotic is a non-digestible feed ingredient that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the gut (25). Thus, for a food ingredient to be considered prebiotic, it must meet three criteria: 1) be resistant to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; 2) be fermented by intestinal microflora; and 3) stimulate selectively the growth and/or activity of intestinal beneficial bacteria(26). These compounds have several beneficial effects on animals and humans and Table 1 summarizes these effects.

Compared to the application of prebiotics in human and pet food, the use of prebiotic in broiler chicken diets does not have a long history. The mechanism of action of prebiotics as AGP replacers is dependent on the nature of the compound and their effects on animal performance will depend not only on the applied dose but also on the bioavailability of the compound and its interaction with the remaining food matrix.

Table 1- Proposed benefits of prebiotics for immunity and health of hosts (reproduced from Shokryazdan *et al*. (26))

Prebiotic effect	Mechanisms/expected results
Changing in the composition and population of gut microflora	-Selectively stimulating beneficial members of the gut microflora with immunomodulatory activity (such as bifidobacteria and lactobacilli) -Inhibiting epithelial adhesion and invasion of pathogenic microbes
Increasing production of fermentation products by gut microflora	-Increasing production of short-chain fatty acids (SCFAs), vitamins, bacteriocins and other antimicrobial compounds. -Butyrate, propionate, acetate and pyruvate are able to beneficially change the expression of cytokines and improve immunity
Simulating intestinal barrier function	-Improving intestinal permeability -Increasing production of mucus

Direct simulation of immune system	-Increasing anti-inflammatory cytokines -Decreasing proinflammatory cytokines -Beneficial effects on G-protein-coupled receptors of immune cells within gut-associated lymphoid tissues and mucosal immune system -Increasing the mucosal Immunoglobulins, altering cytokine and lymphocyte expression, increasing the secretory Immunoglobulin A
Improving nutrients absorption	-Improving small intestine development -Increasing villi height, crypt depth and number of goblet cells per villus

A variety of non-starch polysaccharides or oligosaccharides have been considered as prebiotics, including manno-oligosaccharides (MOS), Fructooligosaccharides (FOS), inulin, galacto-oligosaccharides, lactulose, lactitol, gluco-oligosaccharides, xylo-oligosaccharide, soya-oligosaccharide, isomalto-oligosaccharide, and pyrodextrins (24). Many trials and reviews are available describing the poultry performance benefits of each of these oligosaccharides (17,24,27,28). The trials most often use oligosaccharides extracted from byproducts that are commercially available. However, there are still few studies that evaluate the poultry performance when byproducts containing oligosaccharides with prebiotic effect are directly added in diet. The introduction of byproducts directly or byproduct components is an advantage in the sense that they promote a circular economy "where the value of products, materials and resources is maintained in the economy for as long as possible, and the generation of waste minimized"(29). In this perspective, it is intended to review the byproducts used for this purpose.

Mannose-based oligomers (MOS) are naturally found in certain plants and the outer cell-wall layer of the yeast (17). The MOS extracted from the outer cell-wall layer of yeasts consists of α(1→2,6)-linked mannose branched with α(1→2) and α(1→3)-linked mannose side chains (30) and their effects at various levels have been studied extensively as a prebiotic supplement in poultry diets. Among these effects are the significant increase in body weight and the improvement in feed conversion efficiency, the increase in intestinal villi height, the improvement immune-competence in the intestine, the alteration of jejunal gene expression and the modulation of intestinal microbiota (24). The modulation of intestinal microbiota occurs in part because the yeast derived MOS have ability to agglutination of gram-negative pathogenic bacteria containing type 1 fimbriae (mannose binding lectins) and these pathogens move through the intestine without colonization instead of attaching to intestinal epithelial cells (31). In most of the extractions of yeast derived MOS the raw material is *Saccharomyces cerevisiae* strains, a byproduct of several industrial fermentation processes, including in bakery and brewing. In brewing industry, *Saccharomyces* yeast biomass is the second major

byproduct, being marketed for feed application in the inactive form (after inactivation by heat) and classified as GRAS (32)..

Palm kernel meal (PKM) is a byproduct from Palm oil industry and is an interesting feed ingredient for poultry due to its availability and low cost (33). This byproduct contains β-mannan that consists of *β*-1,4 linked mannose (34). PKM supplementation increase bird productivity and improve chicken health as a consequence of an improvement of the immune system, a reduction on pathogenic bacteria, such salmonella strains, and an increase of non-pathogenic bacteria population of in intestine (34). Besides that, feed digestibility and feed efficiency can be increased when mannan degrading enzymes are included in the diet and MOS are formed. However, there are a few studies that indicated opposite effects such as Adrizal *et al*.(35) that reported that PKM did not affect egg production, feed conversion, or egg weight with or without enzyme supplementations.

MOS and FOS are the most used prebiotics in poultry nutrition(28). FOS and inulin that are mainly composed of fructosyl units (F) bound by β linkage at the position of sucrose (GF). FOS are oligomers, also known as short-chain fructans or polyfructosyl sucroses, with degrees of polymerization (DP) up to 12 while inulin has a higher degree of polymerization. Both these sugars occur naturally in many plants as a storage material and they have been shown to possess significant prebiotic effect (36). Ricke (37) and Buclaw (38) reviewed the benefits of these prebiotics, respectively, in the chickens feeding. Inulin-type fructans (FOS and Inulin) are industrially extracted from the roots of chicory (*Cichorium intybus*) and tubers of Jerusalem artichoke (*Heliantus tuberosus*) (38), however FOS are often obtained by bio-enzymatic transformation from sucrose sources such as sugar beet (39). Chicory roots have a better crop yield of inulin than Jerusalem artichoke and are easy to produce (40). Inulin-type fructans extracted from chicory roots have a beneficial effects on microbial community in the gastrointestinal tract, host health (intestinal morphology, reduction of colonization of pathogenic bacteria) and chickens performance (feed efficiency, body weight gain, nutrients digestibility and absorption, and fat metabolism) (39). When inulin (5 g of inulin/kg of diet) derived from chicory (*C. intybus L*.) roots was added in chicks diet, the hepatic transcriptome profiles was significantly modified, processes and pathways with putative involvement in the chicken growth and performance and the immune status of animals was reinforced (41). When chicory root power were directly incorporated into the broiler chickens diet (2-3%) beneficial effects were denoted: increase of the villus length (VL), villi number ($p < 0.05$), VL/ crypt depth ($p < 0.01$) and villus surface area ($p < 0.02$), enhancing food digestion and absorption, which was reflected in an improvement in feed intake (FI), feed conversion rate (FCR) and body

weight gain (BWG) in broilers (42,43). There are, however, some studies where no significant differences in BWG, FI, FCR were denoted in chicory root powder same doses (43).

Xylo-oligosaccharides (XOS) and arabinoxylo-oligosaccharides (AXOS) have also been widely studied as possible prebiotics in chicken diets, having been shown to be a source of beneficial effects on the health and performance of chickens (44–50). Xylo-oligosaccharides (XOS) are chains of *β*-1,4-linked D-xylopyranoside units and when units of α-L-arabinofuranosyl (Ara*f*) are attached mostly as single substituents, XOS is named arabinoxylo-oligosaccharides (AXOS). These prebiotics can be extracted from several agricultural residues/ byproducts (corn cobs, tobacco stalks, wheat straw, etc) by different approaches (chemical, enzymatic or combination of chemical and enzymatic methods), being these processes still investigated and improved (51). Alternatively, the XOS can be produced *in vivo* by the addition of xylanase to chicken cereal-based diets since the cell walls of monocotyledonous plant species are particularly rich in arabinoxylans, thus increasing the nutritional value of these diets (46). When xylanase is combined with *β*-glucanase (*β*-glucans are also abundant polysaccharides in cereal fiber) lower intestinal viscosity of broilers, better nutrient utilization and better chicken performance are denoted compared to the isolated use of these enzymes (52,53).

In the last years polysaccharides obtained from mushroom wastes have also been investigated as prebiotic. β-Glucans (homo-glucans, and heteroglucans) are the main carbohydrates responsible for this effect (54) and the most common type of this polysaccharides consists of a backbone with β-D-glucose (1 → 3)-linked frequently branched at *O*-6 by β-D-glucose residues (55). In addition to β-glucans, other polysaccharides are present in mushrooms, such as heterogalactans ((1→6)-linked-D-galactopyranosylresidues, which may be substituted at C-2 either by L-fucopyranosyl, D-mannopyranosyl, D-galactopyranosyl or 3-*O*-α-D-mannopyranosyl-α-L-fucopyranosyl residues) and heteromannans which can be galactomannans, glucogalactomannans (main chains composed of (1→2)- and (1→6)-linked-D-mannopyranose) and xylomannas (a main chain composed of (1→3)-linked-D-mannopyranose) (56). Chou *et al*.(54) used mushroom wastes from *L. edodes* stipe (LES), *P. eryngii* base (PEB), and *F. velutipes* base (FVB) to obtain polysaccharides extracts, and they investigated their prebiotic effects on *Lactobacillus acidophilus*, *Lactobacillus casei, Bifidobacterium longum, Streptococcus thermophilus,* and *Lactobacillus delbrueckii* subsp. *bulgaricus in vitro* systems. They found that all the extracts of polysaccharides can enhance the survival rate of these probiotcs even at relatively low concentration (0.1% to 0.5%). Dietary supplementation of 0.1% β-1,3–1,6-glucan of *Schizophyllum commune* enhanced the chicks' host defense to *Salmonella* sv. Enteritidis invasion through a macrophage-modulating mechanism and possibly physical protection of β-glucan in the intestine (57). Willis *et al*.(58) denoted that the continual

administration of mushroom aqueous extract, rich in β-glucans, to broiler chickens enhanced fecal shedding of bifidobacteria and early intermittent administration of the extract decreased *Salmonella* populations. In addition, the broilers that were supplemented with polysaccharide extracts of two mushrooms at 0.5 – 4 g/kg of diet, *Lentinus edodes* (LenE) and *Tremella fuciformis* (TreE), did not present significant diferences in BWG, FI and FCR in relation to the antibiotic group (20 mg/kg, virginiamycin) (59). These data appear to be promising but further studies must be done using mushroom wastes to test whether these wastes have a beneficial effect when incorporated directly into chicken feeds.

Recently, pectic oligosaccharides (POS) have been identified as emerging prebiotics. These oligosaccharides are also called pectin-derived oligosaccharides once they can be produced by chemical, enzymatic and hydrothermal from pectin (60). Pectic polysaccharides are composed of homogalacturonan (α-1,4-linked galacturonic acid residues) and rhamnogalacturonan (repeted units of (α1→2)-rhamnopyranose-(α1→4)-galacturonic acid) branched with arabinans, galactans, xylans, and arabinogalactans (61). The hydrolysis of pectic polysaccharides can produce several type of POS with a variable degree of polymerization and sugar composition, depending on the structure of polysaccharide. The most common and well known POS are arabinogalacto-oligosaccharides, arabinoxylo-oligosaccharides, arabino-oligosaccharides, galacto-oligosaccharides, oligo-galacturonides and rhamnogalacturonanoligosaccharides (61). The agricultural residues, such as orange peel waste (62) or olive byproducts (63), are natural sources of pectic polysaccharides. *In vitro* studies showed that POS can beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, such as Bifidobacteria and Lactobacilli and inhibit cell invasion (64). There are no studies that evaluated the effect of POS supplementation in animal's diets.

2.3.2 Antimicrobials of plant origin / phytobiotics

Phytobiotics are plant derived natural bioactive compounds that have beneficial effects on health and performance of animals when incorporated into feeds (24). They can be broadly classified as herbs (flowering, non-woody, non-persistent plants from which leaves and flowers are used), botanicals (whole or processed parts of a plant, for example, roots, leaves, bark), essential oils (EOs; volatile lipophilic substances obtained by cold extraction or by steam or alcohol distillation) and oleoresins (extracts obtained by non-aqueous solvents). The active compounds of phytobiotics can include terpenoids (mono- and sesquiterpenoids, steroids, etc.), phenolic compounds, and alkaloids (22,28).

Many plants have beneficial multifunctional properties derived from their specific bio-active components and there is the challenge of identifying and quantifying these compounds and characterize their mode of action on animal physiology and health status (22). Around the world, phytobiotics have been investigated as natural alternatives to AGPs in poultry industry, being these plant-derived products recognized as GRAS (9). In Europe, the first botanical feed additive for improving performance in broilers has already been approved, being one commercial blend of phytonutrients containing carvacrol, cinnamaldehyde, and oleoresin *capsicum* (24).

In recent years, a wide variety of herbs and botanicals (thyme, oregano, rosemary, marjoram, yarrow, garlic, ginger, green tea, black cumin, coriander, stevia and cinnamon) and EOs (EOs from clove, coriander, star anise, ginger, garlic, rosemary, turmeric, basil, caraway, lemon, and sage) have been tested either individually or as blends in poultry as alternatives of AGPs (24). The beneficial effects achieved with the incorporation of such products can often also be achieved with the incorporation of agrofood byproducts, since certain phytochemicals may also be present in them (for example, olive byproducts are still rich in polyphenols (65) and *Pinus radiata* bark is rich in proanthocyanidins (66)). There are still not many byproducts whose phytobiotic effects have been evaluated when incorporated into chicken diets. Table 2 summarizes some of these studies. The beneficial effects depend greatly on the composition of the active ingredients in the byproduct being used. When these phytobiotics are applied in animal feed at industrial level, it is necessary to take into account that this composition may suffer oscillations due to biological factors (plant species, growing location and harvest stage), manufacturing processes (extraction / distillation) and conditions during storage (temperature, light, oxygen level and time) (22).

In general, the beneficial effects of phytobiotics are attributed to their antimicrobial, antioxidant and immunomodulatory properties. The inclusion of byproducts in chicken feeds may summarily generate the following phytobiotic effects (as can be seen in Table 2): decreases the mortality rate (67–69); favorably alters the microbial population and reduces the levels of pathogenic microorganisms and toxic metabolites produced (70); improves the body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) (65,67,69,71); reduce oxidative stress and increase antioxidant activity in various tissues and in serum and egg yolk (72–75); ameliorate the negative effects of heat stress (75); improve gut histology, increase villi height and thus expand absorptive surface of the intestine (70); and increased proliferation of immune cells, elevated expression of cytokines, and increased antibodies (66,75,76).

Table 2 - Summary of studies that evaluate the phytobiotic effects resulting from the incorporation of byproducts into chicken diets (oral administration)

Byproduct Animal/ experimental period	Potential phytobiotics	Dietary treatment	Major findings	Reference
Ginger root powder / 675 Hy-Line brown laying hens at 27 wk of age / 70days	Zingiberene, gingerol-related compounds and diarylheptanoids	5 treatments: 0 (control), 5-20 g of ginger root powder/kg of diet	-Laying performance improvement (P = 0.024); -Increased antioxidant status of serum and egg yolk ($p<0.05$); -The optimum supplementation rate of ginger powder appeared to be between 10 and 15 g/kg of diet.	(72)
Ginger (*Zingiber officinale*) Waste Meal (the residues left after super critical fluid extraction to produce ginger oil, ginger oleoresin and ginger total extract)/ 240 coloured synthetic broiler chicks / 42 days	Zingiberene, gingerol-related compounds and diarylheptanoids	4 treatments: 0 (control), 5, 10 and 15% of Ginger Waste Meal	-The BWG and FI were increased ($P<0.05$), however, FCR and carcass characteristics were similar in all the groups.	(71)
Sweet orange (*Citrus sinensis*) peel extract (SOPE)/ 300 male chicks (Ross-308) /42 days	Flavonoids, vitamin C and vitamin E	3 treatments: 0, 1000 and 1250 ppm of SOPE	- Antibody titer response to sheep red blood cells (SRBC), immunoglobulin G and immunoglobulin M were increased in the group fed 1250 ppm of SOPE ($P < 0.05$)	(76)
Orange and banana peel/ 250 healthy Hubbard broiler chicks / 35 days	Orange peel: Flavonoids, vitamin C and vitamin E (76) Banana peel: Phenolics, Carotenoids, and fiber (77)	5 treatments: 0 (control), 1.5% and 3 % of Orange peel and 1.5 and 3% of banana peel	-Better BWG, FCR, dressing % and also lowest mortality (6%) was recorded in chickens supplemented with 3% banana peel ($p<0.05$)	(67)

Pinus radiata bark / 40 Specific-pathogen-free 1-d-old White Leghorn chickens / 35 days	Proanthocyanidins	4 treatments: 0 (control),5, 10, and 20 mg/kg of BW of proanthocyanidin-rich extract (PAE) from *Pinus radiata* bark	-Increased the proliferation of peripheral blood mononuclear, splenocytes, bursal, thymocyte cells -Increased of production of the T helper 1 cytokine (interferon-γ); -Suppression of production of the T helper 1 cytokine (IL-6); -Interleukin-18 mRNA is increased in peripheral blood mononuclear cells.	(66)
Grape byproducts/ 100 1-d-old male broiler Cobb chicks / 21d	Monomeric phenolic compounds such as (+)-catechins, (–)-epicathechin and (–)-epicatechin-3-O-gallate, and dimeric, trimeric, and tetrameric proanthocyanidins	4 treatments: antibiotic-free diet, a positive control (50 mg/kg of avoparcin), and antibiotic-free diets containing grape pomace concentrate (60 g/kg) or grape seed extract (7.2 g/kg).	-Compared with the antibiotic-free diet, the supplementation with antibiotic, grape pomace concentrate and grape seed extract increased the populations of *Enterococcus* and decreased the counts of *Clostridium* in the ileal content. -Animals fed grape byproducts showed a higher biodiversity degree than those fed control diets. -The highest villi height:crypt depth ratio corresponded to birds fed grape pomace concentrate and antibiotic diets	(70)
Grape pomace concentrate / 180 one-day-old male broiler chicks (Cobb strain) / 42 days	Monomeric phenolic compounds such as (+)-catechins, (–)-epicathechin and (–)-epicatechin-3-O-gallate, and dimeric, trimeric, and tetrameric proanthocyanidins (70)	5 treatments: control corn-soybean diet (basal diet), basal diet + vitamin E (200 mg/kg of *α*-tocopheryl acetate), basal diet + 15 or +30 or +60 g/kg of grape pomace concentrate	-Antioxidant activity in diet, excreta, ileal content, and breast muscle were increased in grape pomace concentrate diets. -The grape pomace concentrate supplementation was equally as effective in antioxidant potential as vitamin E.	(73)
Dried tomato pomace / 352 one-day-old male Arian broiler chicks / 42 days	Folate, vitamin C, α-tocopherol, phenolic compounds, and carotenoids (such as lycopene, β-carotene)(75,78)	3 treatments: 0, 3 % (containing 420 mg lycopene/kg diet) and 5 % (containing 708 mg lycopene/kg diet) of dried tomato pomace	-Dietary supplementation of 5% of dried tomato pomace attenuated the detrimental effects of heat stress on the activities of serum enzymes, oxidative status, immune response, and bone composition, but did not influence growth performance under heat stress	(75)

Green tea byproduct / 140 Ross broilers at 1 day old / 42 days	Polyphenolics, especially a distinctive catechin component epigallocatechin gallate	5 treatments: antibiotic free group (basal diet as a control), antibiotic added group (basal diet+0,05% chlortetracycline) and 3 green tea byproduct groups (basal diet+ 0.5% or 1% or 2% of GTB)	-No significant differences were observed in feed intake, feed efficiency, blood LDL cholesterol and docosahexaenoic acid content and cholesterol content in chicken meat among treatments ($p>0.05$) -TBA in chicken meat decreased in groups fed diets with green tea-by product and antibiotics compared to control group ($p<0.05$)	(74)
Olive oil industry waste / 297 22-day-old fast growing (Ross 308) female chicks /	Polyphenols such as tyrosol and hydroxytyrosol, followed by verbascoside and pinoresinol	3 treatments: 0 (control), 82.5 g/Kg and 165.0 g/Kg of olive cake (olive cake is rich in bioactive compounds, such as polyphenols, obtained by mechanical extraction from destoned olives)	- Chicken growth rate increased with increasing olive cake concentration in the diet. -The antioxidant status and the oxidative stability of meat were positively affected, especially when the highest level of olive cake was applied.	(65)
Guava (*Psidium guajava*) leaf meal / 180 day-old broiler chick (Cobb 500) / 42 days	Flavonoids such as guajaverine and psydiolic acid	4 treatments: 0%, 2.5%, 3.5% and 4.5% of guava leaf meal after treating by means of some physical and chemical processes (guava leaf was boiled in water for 1h, boiled in alkaline solution 0.1 N for 1h, boiled in acid solution 0.1 N for 1h, and autoclaved for 20	- FI, BWG and FRC at different dietary treatments were statistically non-significant different among groups of birds. - Fat content and mortality rate were decreased with increased level of guava leaf meal up to 4.5% level.	(68)

		minutes at 15 IP Pressure)		
Seaweed byproducts / 750 of one-d-old Ross male chicks / 35 days	Sulfated polysaccharides (sulfated fucans and alginic acid), phlorotannins, terpenes and peptides (69,79,80)	5 treatments: a) Basal diet (control), b)basal diet+0.5% brown seaweed byproduct, c) basal diet+0.5% seaweed fusiform byproduct, d) basal diet+0.5% fermented brown seaweed byproduct, and e)BD+0.5% fermented seaweed fusiform byproduct	- BWG and FRC of seaweed byproduct groups were clearly higher and the mortality rate was significantly lower, when compared to those of control diet group ($p<0.05$). -The serum immunoglobulins varied with seaweed byproducts: immunoglobulin A antibody content was significantly higher in brown seaweed, seaweed fusiform and fermented seaweed fusiform supplemented groups ($p<0,05$); immunoglobulin M antibody content increased significantly in all seaweed byproducts groups ($p<0,05$); and immunoglobulin G decrease significantly in all seaweed byproducts groups ($p<0.05$).	(69)

Although the aromatic plants are described as source of EOs with phytobiotic effects, there are few studies concerning the incorporation of their byproducts in poultry diets.

3. Onion and its byproducts

3.1 Onion production and consumption

Onion *(Allium cepa* L.), botanically included in the Liliaceae family, is a bulb plant of great economic importance since it is widely used in human food. Its sensory properties coupled with its versatility have made onion a common ingredient to many dishes and acceptable by almost all traditions and cultures (81). Its beneficial effects have been reported by several studies and extensively reviewed, including hypocholesterolemic, hypolipidemic, anti-hypertensive, anti-diabetic, antithrombotic, anti-hyperhomocysteinemia, antimicrobial, antioxidant, anticarcinogenic, antimutagenic, antiasthmatic, immunomodulatory and prebiotic activities (81,82). These properties relate to the presence of several bioactive compounds (82).

Onions are classified based on their color into yellow, red and white and based on their taste as sweet or non-sweet (83). Its grown is widely spread and exhibit a great diversity in form, color, shape, dry matter content and pungency. The onion is marketed in various forms: fresh and processed. The value of processed onion product has increased to meet consumer's demands and it is mainly in the form of dehydrated onion, canned onion and onion pickle (84).

The consumption of this vegetable has increased steadily, reaching a global production of over 88 million tons in 2014 (Figure 1), making this one of the largest horticultural crops in the world (2). In Europe, the countries that most produce onion are the Netherlands and Spain, contributing to almost for an half of the production (45%) (85).

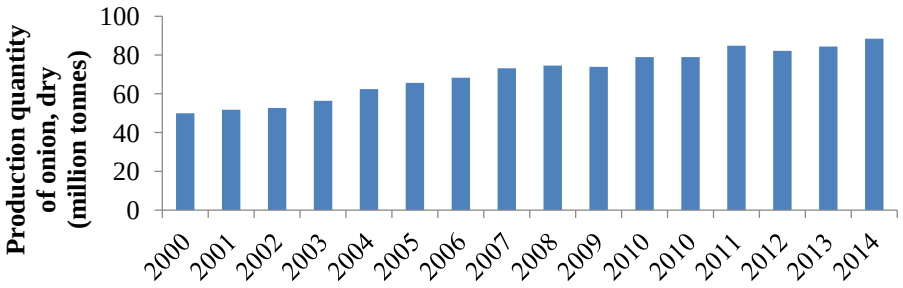

Figure 1 - Onion production levels worldwide over the years (graph built with FAO data(2))

Onion is part of the Mediterranean diet [3] and, in 2016, in Portugal, its apparent consumption reached 123 thousand tons, 26% more than in 2011 (86).

3.2 Onion byproducts

Large amount of onion waste is produced by consumption of onion both domestically and industrially. There is a growing demand for processed onion consumption and industrial

onion residues have been increasing in parallel. About 10 % of all harvested onions are discarded or cheaply sold in the market because they failed to meet the quality standards required by customer (84). In the European Union, more than 500 000 tons of onion waste (residues and surpluses) are produced each year, mainly from Spain, United Kingdom and Holland, while Japan and California produce about 144 000 and 100 000 tons, respectively (84,87,88). Taking account total production of onions in Portugal (86) using the onion byproducts rate from European Union (0.2%), it is estimated that 32 thousand tons are produced per year in Portugal. The main onion wastes include onion skin, two outer fleshy scales, top and bottom (including the roots) generated during industrial peeling (Figure 2), and undersized, malformed, diseased or damaged bulbs (89,90).

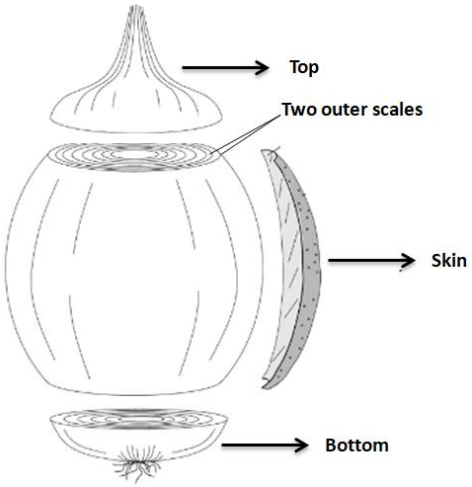

Figure 2 - Parts of the onion bulb considered as solid wastes (reproduced from Zabot *et al*.(89))

3.3 Composition of onions and their byproducts

Onion is a rich source of several component groups, including valuable phytonutrients, such as dietary fiber (DF), fructooligosaccharides (FOS), alkyl or alkenyl cystein sulphoxides (ACSOs) and other sulphur compounds (91). However, its composition is variable and depends on cultivar, stage of maturation, environment, agronomic conditions, storage time and bulb section. The weight of fresh onion is made up of 80–95% water and up to 65% or more of the dry weight may be in the form of non-structural carbohydrates, which include glucose, fructose, sucrose and FOS (90).

Several studies have been carried out to gain knowledge of the detailed chemical composition of the onion and the various byproducts and its conclusions will be presented in detail below.

3.3.1 Nutritional parameters

The nutritional parameters of whole onions and their industrial wastes were evaluated by Benítez *et al*. (90). Dry matter (DM) content is an important quality parameter for the onion industry and the several industrial wastes exhibit differences within this parameter, with the brown skin having the highest value (52 %) (90). The total ash in brown skin is about twice the value reached in the inner (5 % of DM) (90). The crude protein also varies in the several wastes but, contrary to what happens with the DM as total ash content, the value of crude protein is presented lower in the onion skin (2 % of DM) (90).

In terms of mineral content there is a great variation of onions among cultivars because the minerals present in the soil depending on where the onion is cultivated. The distribution of minerals in onion wastes depended on the mineral: elements like magnesium, iron, zinc and manganese were found in top bottom, whereas potassium was found in inner scales. The highest concentration of calcium was found in brown skin and this concentration is about ten times higher than in the inner scales (90,92).

3.3.2 Lipids

The fatty acids profile of domestic onion wastes and bulb is shown in Table 3. The onions contain saturated and unsaturated fatty acids, being its ratio approximately 50:50 both for the onion bulb and top-bottom oils. Outer scale oil presented unsaturated fatty acids in higher quantity about 77% of the total fatty acids. Onion bulb and top-bottom oils have about 40% of oleic acid that had been reported to be beneficial in reducing the risk of cardiovascular diseases. Outer scale oil, in turn, has about 53% of linoleic acid which belongs to the group of dietary essential fatty acids (EFA) and cannot be synthesized synthetically (84,92). As far as we know, there have been no reported studies with the fatty acid profile of onion skin.

Table 3 – Fatty acid composition of the oil extracted from onion bulb and its parts (%)(reproduced from Bello *et al*.(92))

Fatty acids	Carbon number	Onion bulb	Top-bottom	Outer scale
Lauric acid	12:0	0.48	0.46	0.94
Myristic acid	14:0	1.59	1.34	1.28
Palmitic acid	16:0	41.20	41.52	9.80
Palmitoleic acid	16:1	0.15	0.30	2.84
Stearic acid	18:0	5.47	6.76	8.81

Oleic acid	18:1	36.5	38.67	17.57
Linoleic acid	18:2	12.65	10.50	52.87
Linolenic acid	18:3	0.85	0.08	2.88
Arachidic acid	20:0	0.67	0.35	0.59
Behenic acid	22:0	-	-	1.23
Erucic acid	22:1	-	-	0.63
Lignoceric acid	24:0			0.54
Total Saturated fatty acids		49.41	50.43	21.42
Total Unsaturated fatty acids		50.60	49.55	76.79

3.3.3 Sulphur Content

The primary sulphur-containing constituents in onion are the S-alk(en)yl-L-cysteine sulphoxides (ACSOs), and γ-glutamylcysteines (Figure 3) (81,82,93,94). The ACSOs are the flavour precursors, which when cleaved by the enzyme alliinase, give rise to the flavour and pungency characteristic of the *Allium* plants (81). In *Allium* species, four different ACSOs have been found and these three naturally occurring in onion: (+)-*S*-methyl-L-cysteine sulphoxide (MCSO), (+)-*S*-propyl-L-cysteine sulphoxide (PCSO) and *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulphoxide (1-PECSO) (Figure 3) (81,82,93,94). 1-PeCSO is the most abundant of the ACSOs often being more than 80% of the total and so it is the main responsible for the majority of the flavour chemistry in onion. γ-glutamylcysteines besides to serve as important storage peptides, are biosynthetic intermediates for corresponding ACSOs (81). γ-Glutamyl-*trans*-(+)-*S*-(1-propenyl)-cysteine sulphoxide is the major peptide component of onions (Figure 3), representing approximately 50% of the potential flavour and odour precursors(81).

Allinase (EC 4.4.1.4) is compartmentalized in vacuole, but when the tissues of onion are disrupted, it hydrolyses the ACSO to give pyruvate, ammonia and the highly reactive sulphinic and sulphonic acids. These acids condense with other and a cascade of reactions and rearrangements are generated. The result is a mixture of over 50 sulphurcontaining compounds including thiosulphinates, thiosulphonates, mono-, di- and tri-sulphides. Among these compounds are present as the intermediate 1-propenyl sulphenic acid which is generated and instantly rearranges to form the sulphine propanethial-*S*-oxide, being this the lachrymatory factor (LF) of onion (81,82).

The differences in flavour precursor levels and pyruvate levels of several onion tissues present in larger quantities in the fresh parts of the onion compared to the other parts, being in the skin where they finds the lower levels (94)(90). Considering the objective of this work,

the presence of low levels of flavor precursors in this onion waste may be an advantage since low levels of flavor compounds will be generated, most probably not detectable by the animals when the skin is incorporated into animal feeds.

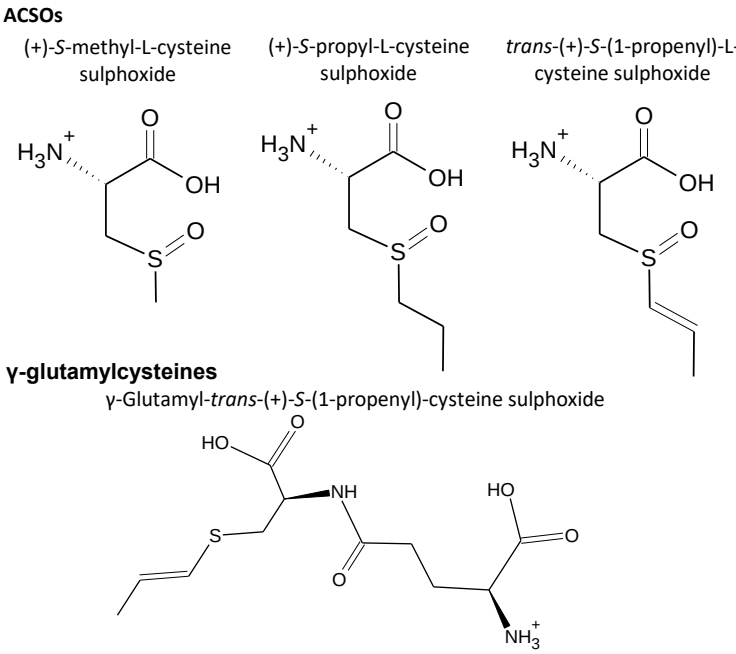

Figure 3 – Chemical structures of main S-alk(en)yl-L-cysteine sulphoxides (ACSOs) and γ-glutamylcysteines of onion

3.3.4 Flavonoids and other phenolic compounds

Flavonoids are present in all terrestrial plants and onion bulbs are among the richest sources of these dietary compounds (81). There are three major groups of flavonoids in onion, the flavonols, the anthocyanins, and the dihydroflavonols and in Table 4 are listed the flavonoids found so far in onion bulbs (95). Flavonols are often found concentrated in the skin of most onions where they impart the yellow/Brown colour, while anthocyanins impart a red/purple color to other varieties (81,95). The main flavonols are based on quercetin (3,5,7,3',4'- pentahydroxyflavone): quercetin is formed by deglucosidation of its glucosides which is then oxidized by a peroxidase in the order quercetin > quercetin 4´-glucoside > isoquercetin > quercetin 3,4´-diglucoside. The structural diversity of the minor flavonols of onions is extensive and includes derivatives of kaempferol, isorhamnetin, and possibly myricetin. The anthocyanins are mainly cyanidin glucosides acylated with malonic acid or nonacylated and the quantitative content of anthocyanins in some red onion cultivars has been reported to be approximately 10% of the total flavonoid content. The dihydroflavonols identified in onions are all based on taxifolin (3,5,7,3',4'-pentahydroxyflavanone) (95).

The onion skin contains approximately six times more total phenolics compounds than fresh outer scales (83). The skin and fresh outer scales of onion contain quercetin derivatives (Figure 4) which account for more than 80% of the total content of flavonoids in onions (83). Quercetin 4`-glucoside and quercetin 3,4`-diglucoside are in most cases reported as the main onion flavonols of the freshy scales, whereas onion skins contain higher concentrations of quercetin aglycon (90). Quercetin are formed by deglucosidation of quercetin glucosides. The browning of the skin onion occurs, during drying in storage, when oxidized products, such as cepaic acid, are formed by autoxidation of quercetin (96). In spite of high-levels of flavonoids in onion skin (2–10 g/kg), much of the onion skins remain unused after processing. To use these wastes, extracts from onion skin were checked for their antioxidant and scavenging activity due to the superoxide radical. (84) In this field Benítez *et al.*(90) showed that antioxidant capacity (FRAP) decreased from the outer to inner part of the onion with a value of 227.8±3.2 µmol Fe^{2+} g^{-1} DM for the onion skin. The fresh outer scale and skin extracts of onion are expected to be a resource for food ingredients, however, food enrichment is justified only when the bioactive components are bioaccessible and bioavailable. There is evidence that onion flavonoids interact with proteins and form indigestible complexes (97) which can affect antioxidant efficacy and protein digestibility.

Table 4 – Flavonoids reported to occur in bulbs of onion (*Allium cepa L.*) (adapted from Slimestad *et al*.(95))

Flavonoids	Range (mg/kg fresh onion tissue)	Reference
Flavonols		
quercetin	1.16 – 749.5	(98–106)
quercetin 4´-glucoside	7.5– 651.0	(102,103,105–114)
quercetin 3,4´-diglucoside	12.7 - 1375	(99,100,102,103,105,107,109–113)
Quercetin 7-glucoside		(95)
quercetin 7,4´-diglucoside	0.2 – 11	(100,102,109–113)
quercetin 3-glucoside = isoquercetin	0.2 – 23	(101–103,109–112)
quercetin 3´-glucoside		(95)
quercetin 3,7-glucoside		(95)
quercetin 3,7,4´-triglucoside	0.9 – 9.0	(100,105,110–112)
quercetin 3-rutinoside	*n. d*. – 18.7	(98,104)
kaempferol	0.54 – 11.57	(101,104)

kaempferol 4´-glucoside		(95)
kaempferol 3-glucoside	*n. d*. – 11.5	(100,103)
kaempferol 7, 4´-glucoside		(95)
kaempferol 3,4`-glucoside		(95)
isorhamnetin 4´-glucoside	3.1 – 358.0	(100,104,109–113)
isorhamnetin 3-glucoside		(111)
isorhamnetin 3,4´-diglucoside	0.8 – 25.0	(100,109–112)
Isorhamnetin		(104)
Myricetin	< 0.4 – 43.9	(115,116)
quercetin dimer		(117)
4´-glucoside of quercetin dimer		(117)
condensation products of quercetin and protocatechuic acid		(117)
quercetin trimer		(117)
Anthocyanins		(95)
cyanidin 3-glucoside	0.01 – 20.6	(100,113,118–121)
Cyanidin 3-arabinoside	10.1 – 11.9	(113)
cyanidin 3-(3´´-glucosylglucoside) = cyanidin 3-laminariobioside	0.03 – 2.39	(100,118–121)
cyanidin 3-(3´´-malonylglucoside)	121.8 – 136.2	(113,118–121)
cyanidin 3-(3´´-malonylarabinoside)	69.4 – 78.6	(113)
cyanidin 3-(6´´-malonylglucoside)	0.67– 17.93	(100,114,119–121)
cyanidin 3-(3´´-glucosyl-6´´-malonylglucoside) = cyanidin 3-(6´´-malonyl-laminaribioside)	0.17 – 11.0	(100,114,119–121)
cyanidin 3-(3´´,6´´-dimalonylglucoside)		(119)
cyanidin 3-(dimalonyl)laminariobioside		(120)
cyanidin 3-(3´´-acetylglucoside)		(118,121)
cyanidin 3-(malonyl)(acetyl)glucoside		(121)
cyanidin 3,5-diglucoside		(119)
cyanidin 3-(malonyl)-glucoside-5-glucoside		(118,121)
cyanidin 4´-glucoside		(122)
cyanidin 3, 4´-diglucoside		(122)
cyanidin 3-(3´´-glucosyl-6´´-malonylglucoside)-4´-glucoside		(122)
cyanidin 7-(3´´-glucosyl-6´´-malonylglucoside)-4´-glucoside		(122)
peonidin 3-glucoside		(118–121)

peonidin 3-(6´´-malonylglucoside)		(118,120,122)
peonidin 3,5-diglucoside		(119,122)
peonidin 3-(6´´-malonylglucoside)-5-glucoside		(122)
delphinidin		(114,120)
delphinidin 3-glucoside		(114)
delphinidin 3-glucosylglucoside	60 - 70	(114)
petunidin glucoside		(114)
petunidin diglucoside		(114)
5-carboxypyranocyanidin 3-glucoside		(123)
5-carboxypyranocyanidin 3-(6´´-malonylglucoside)		(123)
Malvidin 3-galactoside or malvidin 3-glucoside		(118)
Dihydroflavonols		
taxifolin 4´-glucoside		(105)
Taxifolin		(106)
taxifolin 7-glucoside		(106)
taxifolin 3-glucoside		(113)

n. d., not detected

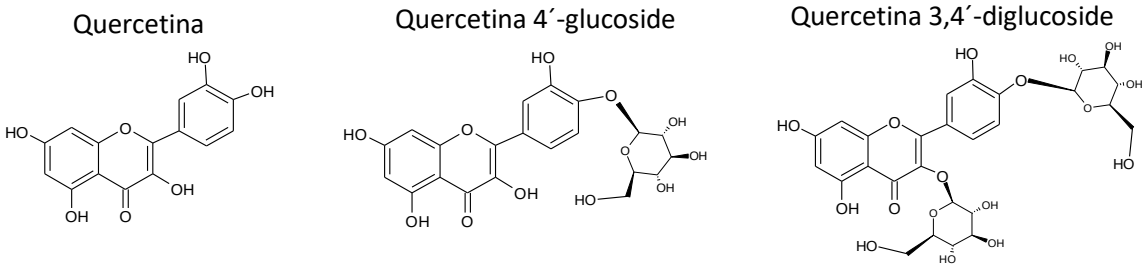

Figure 4 – Chemical structures of the main flavonoids of onion skin (*Allium cepa L.*)

3.3.5 Dietary Fiber

Onion is reported to be a source of dietary fiber (DF), just like other vegetables, and there are already some studies that indicate that onion wastes are also rich in this type of phytonutrients. The dietary fiber of onion tissues in different varieties of onion is cultivar dependent and present different quantities of DF content throughout the different layers of the onion: the highest levels were present in the skin of the onion (68.3 % DM) and the lowest levels were found in the inner part of the onion (11.6 % DM) (16). Soluble/insoluble fiber ratios are important from both dietary and functional perspectives. Generally, the soluble fiber

content of the onion was found to be considerably lower than the insoluble fiber content of the onion. Each part has a different insoluble:soluble ratio and it decreased from the inner to the outer tissues. The IDF:SDF ratio indicates that inner tissue would be more suited for use as a food ingredient, however the outer and skin tissues consisted of the greatest amount of dietary fiber and had the most potential as a fiber ingredient.

In addition the sugar composition profile from IDF, SDF and total DF were studied, the IDF of whole onion mainly comprised glucose (mainly from cellulosic material) and uronic acids, followed by galactose, whereas xylose, mannose, and arabinose appeared in minor amounts (16). Cellulose, xyloglucans, and pectic polysaccharides were the main constituents of IDF of all tissues, but some differences among them were noticed. The uronic acids:neutral sugar ratio in IDF of fresh tissues is a lower as compared to Brown skin and its mean that fleshly tissues were formed by highly branched pectic polysaccharides, whereas in brown skin there was a predominance of homogalacturonans due to the action of galactosidases (16).

The SDF fraction of whole onions showed the presence of uronic acid and galactose as the main sugar constituents, with a trend in uronic acids:neutral sugar ratio increase from inner to outer tissues, indicating that higher amounts of rhamnogalacturonans substituted with galactans were found in fleshy tissues (16).

The characterization of dietary fiber of onion wastes is important in the sense of its biotechnological application. By understanding its composition, chemical, biochemical, or physical treatments can be planned in order to modify DF characteristics of onion waste and improve the SDF:IDF ratio and thus creating the possibility of exploring them as fiber-enriched products.

3.3.6 Non-structural carbohydrates

The predominant non-structural carbohydrates found in onion are sucrose, glucose, fructose and FOS, while starch and raffinose are absent (124).

FOS constitute the main carbohydrate reserve of onion (36). FOS accumulates during bulbing and are then catabolised during regrowth and sprout development of the bulbs. The enzyme responsible for the hydrolysis of them in plants is fructan exohydrolase (FEH, EC 3.2.1.80) and is located in the vacuoles of the cells, being also this the location of the FOS (124). Shiomi *et al.*(36) presented a comprehensive review of metabolism of FOS in onion bulbs.

Today it is known that FOS may have functions such as protecting plants against water deficit by drought or low temperature, and as osmoregulators (124).

Onion bulb has highest content of FOS, the Table 5 presents the FOS so far found and among these stand out kestose, nystose, and fructofuranosylnystose (88,90,125) (Figure 5) which are the only fructose oligomers commercially available (125).

Table 5 - The different fructooligosaccharides of onion bulb separated by HPAEC (adapted from Shiomi *et al*. (36))

Common name	Systematic name
Kestose	1^F-β-D-fructofuranosylsucrose
Neokestose	6^G-β-D-fructofuranosylsucrose
Nystose	1^F(1-β-D-fructofuranosyl)$_2$sucrose
GF3	6^G(1-β-D-fructofuranosyl)$_2$sucrose
GF3	1^F, 6^G -di-β-D-fructofuranosyl sucrose
Fructofuranosylnystose	1^F(1-β-D-fructofuranosyl)$_3$sucrose
GF4	6^G(1-β-D-fructofuranosyl)$_3$sucrose
GF4	1^F(1-β-D-fructofuranosyl)$_2$-6^G-β-D-fructofuranosyl sucrose
GF5	1^F(1-β-D-fructofuranosyl)$_2$-6^G(1-β-D-fructofuranosyl)$_2$sucrose
GF5	1^F(1-β-D-fructofuranosyl)$_4$sucrose
GF5	6^G(1-β-D-fructofuranosyl)$_4$sucrose
GF5	1^F(1-β-D-fructofuranosyl)$_3$-6^G-β-D-fructofuranosyl sucrose
GF5	1^F-β-D-fructofuranosyl-6^G(1-β-D-fructofuranosyl)$_3$ sucrose
GF5	1^F(1-β-D-fructofuranosyl)$_2$-6^G(1-β-D-fructofuranosyl)$_2$ sucrose
GF7	1^F(1-β-D-fructofuranosyl)$_m$-6^G(1-β-D-fructofuranosyl)$_n$sucrose (m+n)=6
	1^F(1-β-D-fructofuranosyl)$_m$-6^G(1-β-D-fructofuranosyl)$_n$sucrose (m+n)≥6

Non-structural carbohydrates decreases from inner to outer tissues and in brown skin small quantities were found. In contrast to others onion byproducts, brown dry outer skin revealed a content of total fructose lower than that of total glucose and has free glucose as the only sugar in significant quantities (3% of dry matter (DM)), indicating the sparse presence of fructans in this tissue. This can happen because SST (sucrose: sucrose fructosyltransferase) and FFT (fructan: fructan fructosyltransferase) activities increase from the outer to the inner tissues bases while fructan hydrolase activity decreases from the outsider to the centre. The SST enzyme catalyzes the conversion of sucrose to kestose and FFT enzyme catalyses the binding of fructosyl units to existing FOS, increasing their degree of polymerization (36). Thus,

the inner scales of the onion present a higher formation of FOS and less hydrolysis of them, compared to the outer tissues (including onion skin).

The total FOS content of whole onion was about 73g /kg on a DM basis (88), *accounting* 68% - 73% of total fructans (90). In onion wastes, FOS contribution to total fructans was among 61 – 87% (90). The content of FOS decreased as the degree of polymerization increased, being kestose the main FOS in whole onion and onion wastes. In brown skin did not detect the presence of nystose and fructofuranosylnystose and only 0.8±0,1 g /kg DM of kestose were detected (88).

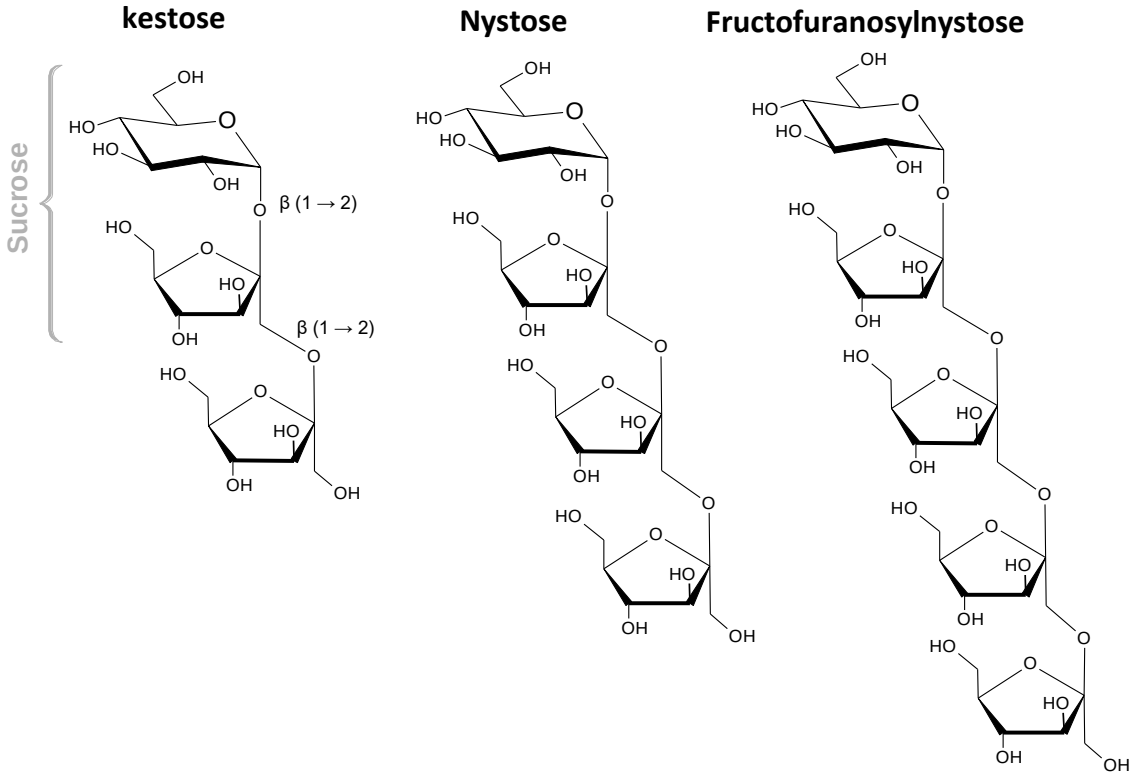

Figure 5 – Chemical structure of kestose, nystose and fructofuranosylnystose

There are already several studies that evaluate the effect of storage and temperature on total fructan and FOS content and the kinetics of these variations in several onion bulb varieties (124–128). These studies are important in the sense that there is the considerable interest in using FOS as food ingredients, being the onion and its byproducts possible sources of these compounds. In the first 8 months of storage, about 75% of onion FOS are degraded (124). FOS hydrolysis is temperature independent, and storage time had more effect on the higher DP FOS than on the lower DP FOS (124,126,127), because they have a relatively high content of fructosyl ends chain.

3.4 Approaches for the use of onion waste

The onion wastes are not suitable for fodder in high concentration due to onion characteristic aroma and neither as an inorganic fertilizer because of the rapid development of phytopathogenic agents such as *Sclerotium cepivorum*. Besides that, their removal by combustion or landfill disposal is rather expensive and cause environmental pollution (88). Hence, there is a need to develop alternative methods to solve this problem: recycling this waste, reducing the environmental impact and increasing the economic value associated with them. There are currently some proposed solutions, however, these are either not yet sufficiently exploited or by themselves do not provide a solution to the massive global production of onion waste.

One of these proposals is to use the onion wastes to capture heavy metals from wastewaters (129) or other hazardous chemicals produced by industry (130) (such as methyl blue (130)) and besides that this wastes have already been proposed as a good source of peroxidases (131–134).

In another biotechnology perspective, these wastes can be used as a nutritive source in microbial conversions and value added products can be produced. However, the fermentation of waste can be hampered by the presence of high concentration of antibacterial and antioxidant substances which might affect the fermentative capacity (135). Bulk mass of rotten or deformed onion, rejected from the industries could be used for onion juice production and this juice could be converted into several useful products such as, bioethanol (136), biomethane (137,138), biogas (137,138), onion vinegar (139) and D-tagatose (136).

Taking into account the proportions with which onion residues are produced today, it is expected that new solutions will have to be created in addition to those that have been presented so far. Therefore, it is necessary to continue to exhaustively study the composition of onion waste in order to find valuable phytochemicals that may have new industrial applications, converting the wastes into useful raw materials, thus promoting a circular and sustainable economy. Onion wastes could be processed and stabilized in order to obtain useful onion byproducts for pharmaceutical, cosmetics, food and livestock industries. For the food and livestock industries, onion wastes can be used not only as a potential source for value-added products but also as food ingredients, although studies in this area are still very preliminary or almost non-existent. Onion wastes can be used as an alternative for synthetic food supplements, which can be an advantage taking into account the consumption demand for natural products (84).

3.5 The prebiotic effect of onion and its byproducts

Onion prebiotic activity is being investigated due to their high dietary fiber content, containing inulin and FOS which are included in the prebiotics classification (25). The carbohydrates present in onion positively influenced multiple parameters in cecum of rats when included in their diets and it was verified an increase in the production of total and individual SCFA (propionate, acetate and butyrate) and a decrease in the pH of the cecal content compared to the control group (140). This SCFA in addition to lowering the colonic pH, have been reported as playing specific roles in the organism: acetate is used as an energy fuel by peripheral tissues, propionate is anti-lipogenic, and butyrate is the primary energy fuel for colonocytes and is related to the reduction of risk for intestinal diseases, as it regulates cell growth, differentiation and apoptosis (140). The direct inclusion of onion powder in juveniles carp diet at 1% increased significantly the population of *Lactobacillus* in gut, being this increase greater when the onion powder was administered together with savory (*Satureja khuzestanica*) (141). These latter studies evaluated the prebiotic effects of onion in animal models, but there is only one study so far reported that evaluates the effects of onion supplementation on chicken diets (142). This study reported that dietary supplementation of 30 g/kg onion increased feed intake and final body weight of broilers at 42 days of age compared to control group and also with antibiotic-treated group (15 mg virginiamycin/kg). Besides that, the birds supplemented with onion presented lower levels of *Escherichia coli* and an increase of *Lactobacilus spp.* population in ileum. The authors associated these beneficial effects with the presence of numerous organic sulphur compounds with antimicrobial activity; however, these effects probably are the combined action of the various bioactive compounds of the onion, among which compounds with prebiotic effect (142).

The prebiotic effects of byproducts of onion have also been investigated. Onion paste as well as the soluble and insoluble fractions had prebiotic effects as evidenced by decreased pH, increased butyrate production and altered rat gut microbiota enzyme activities, besides proving that the onion byproducts have no *in vivo* genotoxicity. Until now, there are no studies on the inclusion of onion byproducts in chicken diets (143).

Prebiotic effect of onion and its byproducts did not prove that this effect is caused solely by theirs carbohydrates such as inulin and FOS. Recently, some researchers have proposed that flavonoids have prebiotic effects as the gastrointestinal bioconversion of flavonoids might stimulate growth of bacterial species such as *Bifidobacteria*. Some species uses the sugar moiety of flavonoid glycosides, others are capable of cleaving aglycons flavonoids, and others are even capable of degrading both structural components (144). As the

onion is a good source of flavonoids (glycosylated and aglycon flavonoids as previously reported), it becomes pertinent to study the prebiotic effects of these onion compounds. Braune *et al*.(145) proposed the mechanism of degradation of aglycon quercetin (one of the main onion flavonoids) by *Eubacterium ramulus,* a strict anaerobe resident in the human intestinal tract. In addition, Ulbrich *et al*. (144) investigate the microbial degradability of the major onion quercetin glucosides by human gut bacteria after thermal treatment and conclude that *Eubacterium ramulus* was capable of degrading quercetin-3,4'-di-O-glucoside and quercetin-4'-O-monoglucoside, while *Flavonifractor plautii* was not. Further studies are still needed to prove that onion flavonol glucosides are effectively prebiotics, namely to study its degradability by other beneficial bacteria (including chickens gut bacteria) and to test its effect *in vivo*.

In addition to these studies, it would still be important to investigate the effects of supplementation of onion or onion byproducts together with phytobiotics on broilers performance and to evaluate if they have a synergistic effect when used together, as well as to define the proportions and quantities that give the best results and possible interactions between compounds of the both additives. Until the date, the prebiotic effect of onion combined with savory phytobiotics was only evaluated in the study of Mousavi *et al*. (141), as previously reported. The composition and properties of essential oils obtained from savory will be discussed in the following chapter.

4. Winter savory and its byproducts

Winter savory (*Satureja montana*), botanically included in the *Lamiaceae* family, is an aromatic plant native to the Mediterranean regions of Europe that often is used as a culinary herb, but also has marked medicinal benefits (146). Savory has been used in cooking since Roman times and now it is used extensively in western Asia, Europe, the Middle East, the USA and Canada (147). The pharmacological and clinical aspects of Satureja species have been reviewed, being among them antioxidant, anti-inflammatory, analgesic and anti-hypercholesterolemic properties (146).

There is increased interest in alternative health products to maintain or improve health in many world geographies, making the market for aromatic and medicinal plants expanding, as well as chemicals and products derived from them (148). Worldwide, the global herbal products market involved 118 billions of dollars in 2012 (149), being that in 2002 the economic value involved had been only 60 billions (150). Portugal also follows this trend, since this type of farms doubled in the period from 2009 to 2012, with an increase in the cultivation area from 80 ha to 180 ha according to the Ministério da Agricultura e do Mar in 2013 (151).

The production and processing of aromatic herbs generates several byproducts depending on the company. Among the byproducts generated are the cuttings of the stems which are separated from the aerial part, since this is the marketable part. In the specific case of the winter savory, the stems represent about 60% of the plant dry weight (152). The valorization of these byproducts in addition to economic benefits, supports sustainability and resource management. One of the approaches to this valorization may be to take away the biologically active constituents present in the plant.

Essential oils are usually obtained from the apical parts of these plants. In this work, essential oils will be obtained from the byproducts (stem shavings), evaluating their composition and antimicrobial properties *in vitro* in an attempt to try to value these byproducts.

4.1 Phytochemical composition of winter savory essential oils (EOs)

Essential oils (EOs), also called volatile or ethereal oils, are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). They are complex mixtures of secondary plant metabolites which are particularly associated with characteristic plant essences and fragrances (18). In nature, EOs play an important role in the protection of the plants as antibacterials, antivirals, antifungals,

insecticides and also against herbivores (153).The steam distillation is the extraction method most commonly used for commercial production of them (154). However, new extraction techniques have been developed to shorten extraction time, reduce organic solvent consumption, improve extraction yield, enhance extract quality, prevent pollution, and reduce sample preparation costs. These techniques include microwave assisted extraction (MAE), supercritical fluid extraction (SFE), and ultrasonic assisted extraction (UAE) (155).

In the particular case of the winter savory, numerous publications have presented data on composition of EOs obtained by hydrodistillation (156–170). The detailed compositional analysis is achieved by gas chromatography and mass spectrometry (GC-MS).

Volatile oils obtained from the aerial parts of *S. montana* by hydrodistillation are quite complex mixtures constituted by several dozens of components (mainly terpenoids and a variety of low molecular weight aliphatic hydrocarbons), and this complexity makes it often difficult to explain their activities. In Table 6 are summarized these chemical components with their respective percentage range in which they occur. The main components of them was the phenolic monoterpene carvacrol (0.4 – 84.2%) and thymol (t – 46.0%), followed by *p*-cymene (t – 41.4%), α-pinene (0.2 - 20.7%), linalool (t – 24.0%), borneol (1.75 – 12.2%), β-cubebene (t – 11.1%), carvacrol methyl ether (1.1 – 11.0%), geraniol (0.1 – 22.3%) and ϒ-terpinene (0.2 – 15.9 %). The chemical structure of these components is shown in Figure 6. The quantitative variability of the EOs is reflected even in the components of higher concentration, as can be seen in the respective percentage ranges, however, there is a greater qualitative variability in relation to the components of less concentration. There is therefore a great variability in the chemical composition of EOs obtained from *Satureja montana* species due to their natural origin, environmental, seasonal and genetic factors (156,158,159,171). EOs obtained from *Satureja montana* L. plants present quantitative variations according to plant development stage (158). Moreover, the same subspecies shows large quantitative variations when grown from ecologically different locations (176). The EOs obtained from S. *montana* subsp. *variegata* cultivated in Sinj (Croatia) have carvacrol as their main compound, whereas EOs obtained from the same subspecies cultivated in Livno (Bosnia Herzegovina) have *y*-terpinene and *p*-cymene as main compounds (in both cases the harvest was made in July). Slavkovska *et al*.(166) confirmed that genetic factors also have an impact on the chemical composition of *S. montana* EO when verifying that different subspecies (*Satureja montana* ssp. *montana* and *Satureja montana* ssp. *pisidica*) present quantitative variations in their chemical composition.

Table 6- Chemical constituents reported to occur in winter savory *S. montana* essential oils obtained by hydrodistillation

Compound	Range concentration (%)	References
	Aliphatic alcohols	
3-Octanol	0.1	(156)
1-Octen-3-ol	0.4 – 1.4	(156,158–164,167,170)
	Aliphatic carboxylic acids	
2-Ethylhexanoic acid	0.1	(156)
n-Hexadecanoic acid	1.0	(156)
	Aromatic compounds	
o-Acetanisole	0.4	(156)
Benzyl benzoate	0.2	(156)
	Monoterpenoids	
Hydrocarbon-type		
α-Thujene	0.2 - 1.8	(157,158,160–164,167–170)
α-Pinene	0.2 - 20.7	(157–171)
β-Pinene	t - 5.5	(158,159,165–169,171)
Sabinene	t-2.25	(171)
δ-3-Carene	t - 0.4	(158,169)
Camphene	0.2 – 2.2	(162,165–169,171)
Myrcene	0.3 – 6.7	(157–169)
α-Phellandrene	0.2 – 1.4	(165,167,171)
α-Terpinene	0.8 – 4.9	(158–165,167–170)
β-Phellandrene	t – 0.4	(158,169)
p-Cymene	t – 41.4	(157–171)
Limonene	t – 17.4	(157–160,162,166–169)
trans-β-Ocimene	0.29 - 0.9	(167,168)
cis-β-Ocimene	1.6 – 6.5	(159,161,166)
ϒ-Terpinene	0.2 – 15.9	(157–171)
Terpinolene	t – 6.3	(158,169,171)
Alloocimene	0.5 – 4.2	(158–161,163,170)
Terpinen-4-ol	t – 10.3	(156–159,162,166–169,171)

Alcohol-type		
trans-4-Thujanol	1.0	(168)
cis-Sabinene hydrate	0.1 – 3.7	(156,161,162,168,169)
trans-Sabinene hydrate	t – 9.3	(156,159,160,164,166–170)
Linalool	t – 24.0	(156–164,166–171)
cis-*p*-Menth-2-en-1-ol	0.1 – 0.7	(156)
cis-*p*-Mentha-2,8-dien-1-ol	t	(156)
trans-*p*-Menth-2-en-1-ol	t – 0.5	(156)
trans-Verbenol	0.2	(156)
Borneol	1.14 – 12.2	(156,158–168,170,171)
p-Cymen-8-ol	0.1 – 8.6	(156,158,169)
α-Terpineol	t – 4.1	(156,159–163,167,169,171)
cis-Piperitol	0.2	(156)
Myrtenol	0.1 – 1.0	(156,159,161)
trans-Piperitol	0.1 – 0.4	(156)
Geraniol	0.1 – 22.3	(156–161,170)
Thymol	t – 46.0	(156–171)
Carvacrol	0.4 – 84.2	(156–171)
Eugenol	t – 0.1	(156)
p-Cymen-7-ol	0.2	(156)
Ether-type		
Carvacrol methyl ether	1.1 – 11.0	(156,158,161–164,170)
Thymol methyl ether	0.5 – 5.1	(156–158,161–164,168,170)
Methyl-eugenol	t	(169)
Methyl thymol	3.2 – 12.8	(160)
Methyl carvacrol	5.4 – 6.4	(160)
Ester-type		
Bornyl acetate	t - 3.86	(156,171)
Isobornyl acetate	t – 0.35	(162,169)
trans-Chrysanthenyl acetate	0.1	(156)
Linalyl acetate	t - 0.7	(167,171)
Carvacrol acetate	0.19	(167)
cis-Piperitol acetate	0.1	(156)
α-Terpinyl acetate	0.1	(156)

Thymol acetate	0.1 – 2.74	(156,158,160,163,164,171)
Geranyl acetate	0.1 – 5.2	(156,158–161,168,170)
Aldehyde-type		
Cumin aldehyde	t – 0.2	(156)
Geranial	t – 5.7	(156,158–160)
Ketone-type		
Thymoquinone	0.1 – 2.8	(156)
2-Ethylmenthone	0.2	(156)
α- Thujone	t – 1.76	(171)
β- Thujone	t - 0.54	(171)
Menthone	0.59	(168)
Carvone	0.41 - 0.66	(157,165)
Dihydroactinidiolide	0.1 – 0.4	(156)
Oxide-type		
Linalool oxide	t – 0.4	(156)
Piperitenone oxide	t	(156)
Eucalyptol	t – 19.5	(162,167,169,171)
Sesquiterpenoids		
Hydrocarbon-type		
Ledene (Viridiflorene)	0.29 – 1.28	(158)
β-Cubebene	t – 11.1	(158–160,163,164)
Calarene	2.5	(159)
α-Copaene	0.1 – 1.8	(156,159)
β-Bourbonene	0.15 – 3.2	(156,157,159,160,167)
β-Elemene	0.1	(156)
Longifolene	t	(169)
β-Caryophyllene	0.1 – 10.68	(156,158,160,162–172)
α-Amorphene	0.3	(167)
β-Copaene	0.1	(156)
Aromadendrene	t – 1.43	(156–158,160,161,163,164)
α-Humulene	0.1 – 0.3	(156–158,160,164,170)
ϒ-Muurolene	0.1	(156)
Germacrene D	0.35 – 1.9	(156,157,167)
β-Patchoulene	0.49	(167)

α-Elemene	0.3	(170)
ϒ-Elemene	0.75	(168)
Bicyclogermacrene	1.0	(156)
β-Bisabolene	0.5 – 1.86	(156–158,160,162,164,167–170)
ϒ-Cadinene	0.1 – 1.0	(156–158,167)
δ-Cadinene	t – 2.1	(156,157,160,161,163,164,167,168,172)
α-Cadinene	0.1	(156)
α-Calacorene	t	(156)
6α-Hydroxygermacra-1(10),4-diene	0.1	(156)
Eudesma-4(15),7-dien-1-β-ol	t – 0.1	(156)
14-Hydroxy-α-humulene	t	(156)
Alcohol-type		
Viridiflorol	0.3 – 5.3	(156,159)
Salviadienol	0.1	(156)
trans -Nerolidol	0.3	(156)
Spathulenol	0.15 – 5.3	(156,158–163,167)
Guaia-6,10(14)-diene-4β-ol	0.1	(156)
Torilenol	0.2 – 0.3	(156)
1-epi-Cubenol	t	(156)
Caryophylla-3(15),7(14)-dien-6-α-ol	0.1 – 0.5	(156)
Caryophylla-3(15),7(14)-dien-6-β-ol	0.4 – 2.2	(156)
Isospathulenol	t – 0.2	(156)
epi-α-Cadinol	t	(156)
β-Eudesmol	0.2	(156)
α-Cadinol	0.5	(156)
14-Hydroxy-β-caryophyllene	0.4 – 2.6	(156)
14-Hydroxy-9-epi-β-caryophyllene	0.8 – 4.1	(156)
Khusinol	0.4 – 0.5	(156)
Farnesol	4.1	(168)
Oxide-type		

Humulene epoxide II	0.3	(156)
Caryophyllene oxide	0.43 – 7.7	(156,157,159–162,167–169,171)
Ketone-type		
Salvial-4(14)-en-1-one	t – 0.2	(156)
3-iso-Thujopsanone	0.2	(156)
Oplopanone	0.1	(156)
Camphor	0.38 – 2.85	(159–162,171)

t, traces (<0.1%)

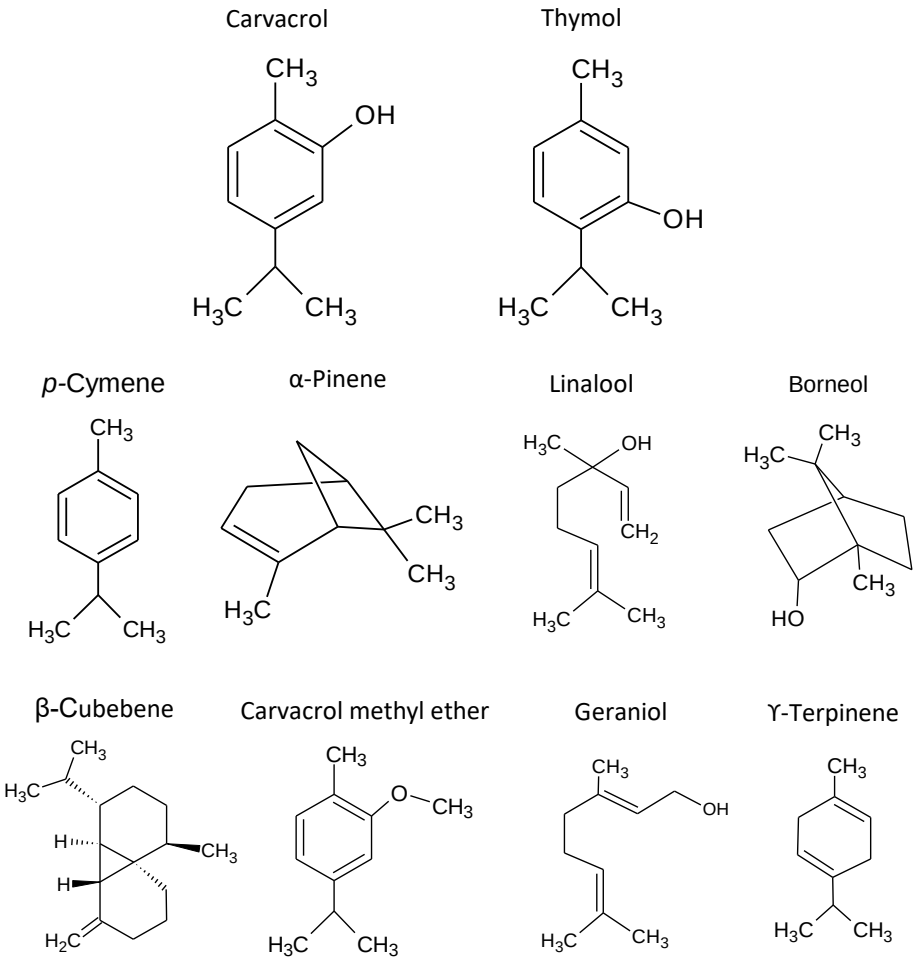

Figure 6 - Chemical structure of some major components of winter savory essential oil

The yield of essential oils obtained by hydrodistillation from *S. montana* aerial parts ranged from 0.5 % to 2.5% as can be seen in more detail in the Table 7. *S. montana* EO was also obtained by microwave assisted hydrodistillation (MW-HD) by Rezvanpanah *et al*. (173) with water/plant ratio of 20:1 (w/w) and the yield (0.7%) is comparable with previous mentioned values by hydrodistillation. When compared to hydro-distillation, optimised microwave treatment can showed an increase in oxygenated compound content as it happened in *Rosmarinus officinalis* EO (174), however, other EOs, as the *Thymus vulgaris L.* EO

(175) or *Mangifera indica L.* EO (155), did not have any influence on composition by microwave irradiation. *S. montana* EO obtained MW-HD has not yet been chemically characterized and compared with the EO obtained by conventional hydrodistillation. As far as we know, there have also been no studies evaluating the composition of the essential oil of the winter savory branches.

Table 7 – Extraction yield of essential oils obtained from aerial parts *Satureja montana* by hydrodistillation (HD) or microwave assisted hydrodistillation (MW-HD).

Essential oil yield %(v/w)	Extraction methodology	References
0.5 -1.7	HD	(156)
2.5	HD	(161)
0.80 - 1.46	HD	(158)
0.7% - 1.9	HD	(159)
0.47	HD	(162)
0.9	HD	(169)
1.4	HD	(170)
1.7	HD	(160)
0.8	HD	(165)
1.56 - 1.70	HD	(167)
0.7	MW-HD	(173)

4.2 Savory EOs in poultry nutrition

The number of studies examining the use of EOs in poultry production has increased rapidly in response to demand for use natural products in the food chain (176). Many of these studies describing various beneficial effects (18,176,177). The mode of action of the bioactive compounds in birds has not yet been fully explained (18), but the EOs actions can be grouped into some categories: sensorial, metabolic, immunologic, antioxidant and antimicrobial.

EOs and their bioactive components act as food condiments and can be detected peripherally in oral and nasal cavities through somatosensing, smell and taste. This sensory perception stimulates digestive secretions and gut motility, preparing the gastrointestinal tract for food reception (18). In broilers, few studies reveled that commercial EOs and blend of EOs enhance the activities of trypsin, of amylase in tissue homogenates of the pancreas, as well as the jejunal chyme content, potentiating an improvement in nutrient digestibility (178).

However, there are no reported studies that have evaluated the effect of savory EO at this level.

Metabolically the EOs present hypolipidemic activity (177). Very few studies study the potential effects of EOs in lipid metabolism in chickens, using the savory oils it is reported that dietary supplementation of 500 mg/kg *Satureja Khuzistanica* essential oil caused a significant decrease in plasma LDL, total cholesterol and triglycerides (179).

Some essential oils positively influence the poultry immune system (180). The addition of thymol + carvacrol (major components of winter savory oils, as discussed above) modified linearly ($P < 0.05$) immune response in broiler chickens by increasing hypersensitivity response, total and immunoglobulin G anti-sheep red blood cell titers, and decreasing heterophil to lymphocyte ratio (181). Heterophils "are predominant granulated leukocyte in acute inflammatory response in gallinaceous birds"(182), being heterophil/lymphocyte ratio used as a measure of stress in chickens(183). The addition of savory (*Satureja khuzistanica*) reduced the adverse effects of aflatoxin B_1 on growth performance and provided slight positive effect on serum biochemistry and humoral immune responses (significantly increased antibody titer against the Newcastle virus) (184).

Many essential oil of plants are known for their antioxidative properties (18,180). The antioxidant mechanisms of EOs are based on both their ability to donate a hydrogen or an electron to free radicals (177). Carvacrol and thymol, the two main phenols, are principally responsible for this activity, and these have the ability to delocalize the unpaired electron within the aromatic structure or hydrogens from their OH groups, thus protecting other biological molecules against oxidation (18,177). This activity the oils have been well investigated since it avoids the lipid oxidation in meat by delaying the formation of the hydroxyl peroxide. Lipid oxidation is a major problem encountered during meat processing, cooking, and refrigerated storage, affecting the quality of the product due to loss of desirable colour, odour and flavour, and shortens shelf-life. The poultry meat is particularly susceptible to oxidative deterioration due to this relatively high content of polyunsaturated fatty acids (18). The antioxidant status of chicken meat can also be enhanced using natural antioxidants in diet of this animals, such as essential oils or their ingredients, as review Adaszyńska-Skwirzyńska *et al*.(180) and Brenes *et al*.(18). The antioxidant activity of the EO of savory (*Satureja montana*) has already been evaluated (156,169,170). The effect of *Satureja montana* EO supplementation under the oxidative stability of chicken meat has not yet been evaluated, but savory *Satureja khuzestanica* EO supplementation decreased omega-3 enriched egg yolk lipids oxidation during refrigeration and room temperature preservation (185).

It has long been recognized that some EOs have antimicrobial properties (18,154,177,178,180). Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antibacterial activity is not attributable to one specific mode of action, but a cumulative effect on many different targets in various parts of the cell (154). Few studies indicated that whole EOs have greater antibacterial activity than their major components and this suggests that minor components are critical to the activity and may have a synergistic effect (18).

EOs and their components are hydrophobic, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable. Subsequently, extensive loss of cell contents or the exit of critical molecules and ions can occur, leading to cell death (154). Generally, the EOs with high contents of phenolic compounds, such carvacrol and thymol, possesse the strongest antibacterial properties against food borne pathogens, since that these compounds have ability of disintegrate the outer membrane of Gram-negative bacteria (18). In addition to this action, it is still reported that carvacrol and other substances prevent the synthesis of flagellin, causing bacterial / cells to be aflagellate / non-motile, and therefore less able to adhere to epithelial cells and potentially less pathogenic.

Most studies that investigated the action of whole EOs suggest that they are more active against Gram-positive than Gram-negative bacteria, however some EOs were effective against both the Gram-positive and Gram negative bacteria (18).

There are already *in vitro* studies evaluating this effect of savory EO on various microbial pathogenic strains. Among the pathogens that were inhibited by savory EO are Gram-positive bacteria, such as *Clostridium perfringens* (162), *Listeria monocytogenes,* (186); *Staphylococcus aureus* (186), and Gram-negative bactéria, such as *E. coli O157:H* (186), *Salmonella* Typhimurium*,* (186), *Pseudomonas aeruginosa,* (160), *Serratia marcescens,* (160). The antimicrobial properties of these essential oils make them capable of modeling the intestinal microbiota. *In vivo* effects of savory EOs were recently evaluated by Masouri et al. (179) that showed that the dietary supplementation of 500 mg/kg *Satureja khuzistanica* EO increased caecal population of *Lactobacillus*, and reduced total bacterial and *Escherichia coli* count.

These EOs activities prevent the onset of diseases and promote the health status in chickens, having impacts on the final quality or productivity. There are still very few studies on the effects of savory EO supplementation in poultry nutrition, and the results are often variable. The variation of the chemical composition of the EOs (due to geographic factors for example) is sufficient to cause variability in the degree of susceptibility of Gram-negative and

Gram-positive bacteria, being one of the explanatory points for these different results. Dietary supplementation of 500 mg/kg *Satureja khuzistanica* essential oil increased feed intake and body weight gain, reduced digesta viscosity and increased villus height, villus height-to-crypt depth ratios and decreased crypt depth of the duodenum (179). There are still no reported studies on the effect of the essential oils of savory winter in poultry diet as an alternative to AGP.

5. Materials and Methods

5.1 Plant material

Dried branches of *Satureja montana* (agroindustrial byproducts) were provided by Ervital - Infusões e Condimentos Biológicos, being harvested in 2016 and 2017 from Serra do Montemuro, Portugal. The samples were transported in bags and stored in the dark at room temperature.

Brown onion skin samples (domestic byproducts) consisted of the dry outer skin of the bulb and dried top onion consisted of old flowering shoot. After their reception, they were milled and the obtained powder was stored at room temperature until further analysis.

5.2 Extraction methodologies

5.2.1 Hydrodistillation (HD)

Satureja montana samples (100g of each individual harvest) were cut into small pieces of two centimeters and mixed with 1L of distilled water. Then hydro-distillation was performed for approximately two hours, when 250 mL of distillate were collected. The condensed vapors do not presented two phases, being in the form of an emulsion. The hydrodistillates were subjected to liquid-liquid extraction procedure for obtaining the essential oil, described below.

5.2.2 Microwave assisted hydrodistillation (MW-HD)

A NEOS-GR apparatus from Milestone (Figure 7) was used for microwave assisted hydrodistillation of *Satureja montana* byproducts. This is a 2.45 GHz multimode microwave reactor with a maximum powder of 900W. Samples (100g of each individual harvest) were cut into small pieces and hydrated with 1L of distilled water overnight. The branches were drained and weighed, in order to infer the percentage of water that was stored intrinsically in the plant material. The overplus hydration water was stored at 4 °C for few hours until further analysis and hydrated samples were placed in the Pyrex extraction vessel and irradiated with 500 W under atmospheric pressure. The hydrodistillate were continuously collected and for each fraction of 10mL were registered the experiment time and the vessel temperature (monitored by an optical fiber sensor). The process was stopped when i) a decrease in the collection flow was denoted, or ii) 85% of the samples water was collected, or iii) it reached 102 ° C in the vessel, avoiding to reach the burning point. Then, the distilled were subjected to the procedures for obtaining the essential oil and the overplus hydration water were subjected to

the same procedures to understand if some of the compounds found in the essential oil would be released in the process of hydration of the samples.

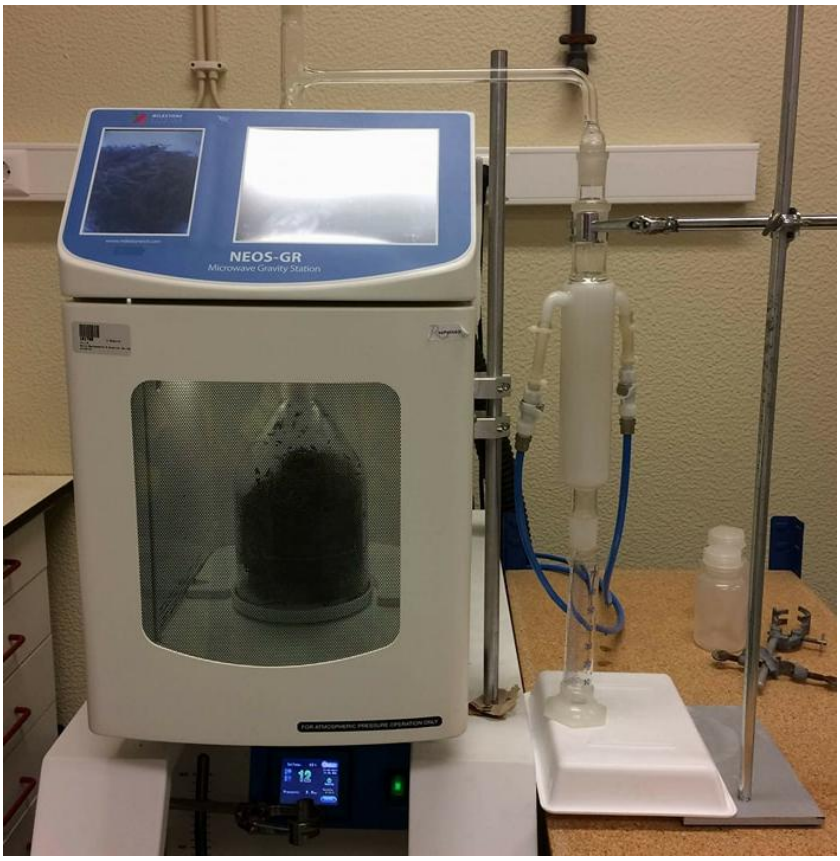

Figure 7 - NEOS-GR apparatus from Milestone

5.2.3 Extraction of essential oils from distillates or from excess hydration waters

The EOs and overplus hydration water extract (OHWE) were extracted with dichloromethane and dried over anhydrous sodium sulphate. After evaporation of dichloromethane in a stream of nitrogen, the EOs and OHWE were stored in vials at 4 °C until used. The extraction yield was determined by gravimetry.

5.2.4 Sequential extraction of the skin and top onion carbohydrates

The skin and top onion sugars were sequentially extracted with ethanol and water. The dried powder of skin and top onion (10 g) were homogenized in 50 mL of 80% ethanol and boiled for 10 minutes. Subsequently the ethanol insoluble residue (REt) and supernatant (SnEt) were separated by decantation and filtration with a pore of a 16 to 40 µm. The Et residue were extracted with hot distilled water (80°C; solid / liquid ratio of 1/50) for 10 min, obtaining the final residue (RAq) and the aqueous supernatant (SnAq) by decantation and filtration using MFV3 glass microfibre filter. All extracts were vacuum-evaporated at 40°C and freeze-dried.

5.2.5 Extraction of the skin onion carbohydrates by microwave superheated water

Skin onion powder (2.14 g) was suspended in 58 mL of extraction solvent in a 100 mL Teflon-coated vessel at a liquid/solid ratio of 27:1. Distilled water and etanol to 65% were the two extraction solvents tested. After one hour of stirring, the slurry was then treated in a microwave oven (MicroSYNTH Labstation for Synthesis; maximum output, 1 kW, 2.45 GHz; Milestone Inc., Shelton, CT, USA), shown in the Figure 8. Microwave powder was adjusted to attain 120, 140 or 160 °C in 2.5 min, and maintain the temperature for 2 min. The temperature and pressure were controlled with a thermocouple immersed in the slurry. For each temperature, two extraction replicates were done.

After microwave assisted extraction, the residue and the obtained extract from each reactor were separated by decantation and filtration using a MFV3 glass microfibre filter. All extracts were vacuum-evaporated at 40°C and freeze-dried.

Figure 8 - MicroSYNTH Labstation apparatus from Milestone

5.3 Essential oils components identification

The EOs obtained by different methodologies (hydrodistillation and microwave assisted hydrodistillation) from different harvest of *S. montana* and OHWRs were analyzed on an Agilent Technologies 6890N Network gas chromatograph (from Agilent Technologies, Inc., USA), equipped with a 30 m length, 0.32 mm of internal diameter and 0.25 µm of film thickness DB-FFAP mass spectrometry (MS) column (123-3232 from Agilent Technologies.

Helium was used as carrier gas (1.7 mL/min) with the column pressure at 3.52 psi. The temperature program used was as follow: initial temperature was 60 ^{o}C, with a linear increase of 2 ^{o}C/min up to 180 ^{o}C, followed by linear increase of 5 °C/min until 220 °C, remaining thus until the end of the run (70 min.). The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range 33–300 *m/z* in a 1 s cycle in a full scan mode acquisition. The injection was performed in splitless mode. Each EO was injected directly after dilution in dichloromethane (1:200) and internal standard (2-undecanol) addition. Each OHWE, in turn, was filtered on a pore filter of 0.45 µm (after dilution in dichloromethane (1:30) and internal standard addiction) prior to injection, in order to remove the compounds that were in suspension. Identification of volatile compounds was achieved by comparison of the GC retention times and mass spectra with those, when available, of the pure standard compounds. All GC mass spectra were also compared with the library data system of the GC–MS equipment (*Wiley 275*) and according to the compounds previously described for *Satureja montana* aerial EO (Table 6). The quantification of the identified compounds were calculated based on GC peak areas, taking into account the concentration of the internal standard added and the calculated response factor correlation between internal standard and eugenol.

5.4 Onion extracts components identification

5.4.1 Total sugar analysis

All extracts obtained from skin and top onion powder by sequential extraction or by microwave assisted extraction were analysed for their sugar content as described by Bastos, Coelho and Coimbra (187). The total sugars were determined by the sum of the individual neutral sugars content analyzed by gas chromatography with flame ionization detector (GC-FID) as their alditol acetates and uronic acids content determined by the 3-phenyl-phenol colorimetric method.

The samples (1–2 mg) were incubated with 72% (w/w) H_2SO_4 during 3 h at 30 °C with occasional shaking and hydrolysed with 1 M H_2SO_4 at 100 °C. After 1 h hydrolysis, it was collected 0.5 mL of solution for uronic acids determination. The neutral sugars released were reduced with $NaBH_4$ (15% in 3 M NH3) during 1 h at 30 °C. The excess of the reducing agent was destroyed by the addition of glacial acetic acid (100 µL) and then the neutral sugars were acetylated, with acetic anhydride (3 mL) and 1-methylimidazole (450 µL), during 30 min at 30 °C. The alditol acetates were separated by liquid-liquid extraction with water and dichloromethane and after evaporation of organic solvent, GC-FID analysis was performed

using 2-deoxyglucose as internal standard in a Perkin Elmer — Clarus 400 chromatograph (PerkinElmer, Massachusetts, USA) equipped with a FID detector and a DB-225 column (30 m × 0.25 mm and 0.15 µm of film thickness, J&W Scientific, Folsom, CA, USA). The samples were diluted in 50 µL of anhydrous acetone and were injected in split mode (split ratio of 20). The temperature of the injector was 220 °C while the detector operated at 230 °C. The oven temperature program was as follows: initial temperature was set at 220 °C, maintaining this temperature for 7 min, and then rises to 240 °C at 5 °C/min. Hydrogen was used as carrier gas at a flow rate of 1.7 mL/min. The neutral monosaccharides present in the samples were identified taking into account the retention times of previously injected standards.

In 3-phenyl-phenol colorimetric method, galacturonic acid was used as standard. To the 0.5 mL of diluted hydrolysed sample (1:4) was added 3 mL of boric acid 50 mM H_2SO_4 98% (w/w). The mixture was manually shaken and the test tubes were heated at 100°C during 10 min. After cooling in a water-ice bath, 100 µL of *m*-phenylphenol was added, reacting 30 min in dark, and the pinkish chromogen produced was measured at 520 nm. The blank of each sample was run without addition of *m*-phenylphenol. The absorbance of the blank sample was subtracted from the total absorbance.

5.4.2 Free sugar content

Free sugar content were also analyzed after reduction and derivatisation to alditol acetates, without previous hydrolysis. The free sugars content were determined by the sum of the individual free monosaccharides, di- and trisaccharides analyzed by GC-MS on an Agilent Technologies 6890N Network gas chromatograph (from Agilent Technologies, Inc., USA), equipped with a 30 m length, 0.25 mm of internal diameter and 0.10 µm of film thickness DB-1 mass spectrometry (MS) capillary column (J & W Scientific, Folsom, CA, USA). Helium was used as carrier gas (0.86 mL/min) with the column pressure at 73.8 kPa. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range 33-700 *m/z* in a 0.1 s cycle in a full scan mode acquisition. The samples were dissolved in anhydrous acetone were injected in split mode (split ratio of 33), with the injector operating at 250 ^{o}C. The temperature program used was as follow: initial temperature was 140 ^{o}C, with a linear increase of 5 ^{o}C/min up to 180 ^{o}C and standing 1 min at this temperature, followed by linear increase of 5 °C/min until 250 °C, maintaining this temperature for 10 min, followed by linear increase of 10 °C/min until 325 °C remaining thus until the end of the run (43 min.).

5.4.3 Glycosidic-linkage analysis

The glycosidic linkages of polysaccharides from onion extracts obtained by sequential extraction were identified by methylation analysis, using the same procedure as Simões *et al*. (188). In this procedure, the partially methylated alditol acetates (PMAAs) were produced by methylation of the free hydroxyl groups, followed by hydrolysis of polysaccharides, reduction and acetylation of hydroxyl group involved in glycosidic linkages; being PMAAs posteriorly separated and analysed by gas chromatography-quadrupole mass spectrometry.

The samples (1-2mg) were placed in a vacuum oven during 10h with the presence of P_2O_5 and were dissolved in 1 mL of anhydrous dimethylsulfoxide (DMSO), stirring overnight at ambient temperature. NaOH pellets (40 mg) powdered under argon were added and the solutions were left under stirring for 30 min. Then, CH_3I (80µL) were added and allowed to react during 20 min with stirring, following by a second addition of CH_3I (80µL) and stirring for another 20 min. Distilled water (2 mL) and dichloromethane (3 mL), were then added, and dichloromethane phase was washed three times by addition of distilled water (2 mL). The organic phase was evaporated to dryness and the material was remethylated. The remethylated material was hydrolyzed with 2 M TFA (1 mL) at 121 °C for 1 h, cooled, and rotary evaporated to dryness. The partially methylated sugars were then suspended in 0.3 mL of 2 M NH_3 and 20 mg of $NaHD_4$ was added. The mixture was allowed to react at 30 °C for 1 h, and the reaction was terminated by the addition of 0.1 mL of glacial acetic acid. The acetylation of the partially methylated alditols was performed by adding 1-methylimidazole (0.45 mL) and acetic anhydride (3 mL) and allowing reaction for 30 min at 30 °C. This solution was treated with water (3 mL) to decompose the excess of acetic anhydride, and the partially methylated alditol acetates (PMAA) were extracted with dichloromethane (2.5 mL). The dichloromethane phase was washed two times with water (3 mL) and evaporated to dryness. The PMAAs were separated and analysed by gas chromatography–mass spectrometry (GC–MS) on the same equipment and column as free sugars. The samples were diluted in 20-30 µL of anhydrous acetone and were injected in split mode (split ratio of 2.0) with the injector operating at 250 °C, and using the following temperature program: initial temperature was 80 ^{o}C, with a linear increase of 10 ^{o}C/min up to 140 ^{o}C, and standing for 5 min at this temperature, followed by linear increase of 0.2 °C/min until 150 °C, followed by linear increase of 60 ^{o}C/min up to 250 ^{o}C, with further 2 min at this temperature. The helium carrier gas had a flow rate of 1.84 mL/min and a column head pressure of 124.1 kPa. The Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range 50-700 *m/z* in a 0.1 s cycle in a full scan mode acquisition.

5.5 Antimicrobial activity

5.5.1 Microorganisms

The antimicrobial activity of EOs was evaluated using two Gram-negative bacteria strains (*Salmonella enterica* sv Anatum SF2, *Escherichia coli* ATCC 25922) and one Gram-positive bacteria strain (*Staphylococcus aureus* ATCC 6538). *Salmonella enterica* sv Anatum SF2 was isolated from seagulls on the island of Berlengas (Peniche, Portugal) while *E. coli* ATCC 25922 and *S. aureus* ATCC 6538 were obtained from the American Type Culture Collection. These strains were cryopreserved in glycerol at -80 ° C. For cell reactivation, 20 µL of cell suspension in glycerol was pipetted to 5mL Luria-Bertani (LB; Nyztech, Portugal) broth and the culture was incubated at 37 ° C for 18-24h with a constant stirring of 180 rpm. The microorganisms used in this study were maintained on Luria-agar (LA) medium (prepared from the mixture of the LB medium with agar (JMGS, Lda., Portugal) at 4 °C. Prior to each bacterial test, subcultures were made by streaking microorganisms on LA medium, which were incubated at 37 °C for 24 h.

5.5.2 Disc diffusion assays

The inocula were prepared in physiological saline starting from one or more colonies of the previously prepared streak for each strain, cell density being standardized with 0.5 Macfarland scale to achieve an inoculums of approximately 10^8 CFU m^{-1}. A portion of this microbial suspension was evenly placed onto the surface of pre-dried Mueller–Hinton agar (MHA;Oxoid; England) plates with the aid of a sterile swab and the plates were allowed to dry for 20 min in the laminar flow chamber. Sterile 6 mm filter paper discs (Liofilmchem, Roseto degli Abruzzi TE, Italy) were placed on the plates and immediately sterile water and EO was added (Table 8). Sterile discs with sterile water or DMSO were used as negative controls and ciprofloxacin (5 µg) (CIP; Oxoid; England) and gentamicin (10 µg) (CIP; Oxoid; England) discs were used as positive controls. After allowing 15 min at room temperature for the essential oils to diffuse across the surface, the plates were incubated at 37 °C for 18-24 h. The diameter of the inhibition zone was measured in millimeters and the assay was carried out three times for each strain. The amount of oil added to each test disc was adjusted over several previous tests (successively lower quantities) for each microorganism. The assay in which it was possible to effectively measure the zone of inhibition for a given microorganism was defined as assay 1. An assay 2 was further carried out in which three successively smaller amounts of oil were pipetted in order to try to infer whether the zone of inhibition has any proportionality with the

amount of bioactive compound present. Table 8 describes in detail which quantities of oil were tested in assay 1 and 2 for each strain.

Table 8 - Description of each test, positive and negative control discs contained in the Disc diffusion assay 1 and 2

Assay 1			
	Test discs	***Negative control disc***	***Positive control discs***
Staphylococcus aureus ATCC 6538	20 µL sterile water + 0.5 µL EO	20 µL sterile water	5µg ciprofloxacin 10µg gentamicin
Salmonella enterica sv Anatum SF2 and E. coli ATCC 25922	20 µL sterile water + 2 µL EO	20 µL sterile water	5µg ciprofloxacin 10µg gentamicin
Assay 2			
	Test discs	***Negative control disc***	***Positive control discs***
S. aureus ATCC 6538	1) 10 µL EO solution[a] 2) 7.5 µL EO solution[a] 3) 5 µL EO solution[a]	10 µL DMSO	5µg ciprofloxacin 10µg gentamicin
Salmonella enterica sv Anatum SF2 / *E. coli* ATCC 25922	1) 20 µL sterile water + 2 µL EO 2) 20 µL sterile water + 1.5 µL EO 3) 20 µL sterile water + 1.0 µL EO	20 µL sterile water	5µg ciprofloxacin 10µg gentamicin

[a] The EO solution was prepared in DMSO (0.05 µL EO/ µL solution)

5.5.3 Microdilution assays

The minimal inhibitory concentration (MIC) values were determined using a broth microdilution assay. Microorganism suspensions were prepared starting from the previously prepared streak for each strain and were adjusted to 0.5 McFarland turbidity standards. Aliquots of the oil under investigation, dissolved in 1% of DMSO, were first diluted to the highest concentration (500 µg/mL) to be tested and then serial two-fold dilutions were made in order to obtain the concentration range from 5 to 500 µg/mL in Mueller–Hinton broth (MH

broth; merck). The MIC was defined as the lowest concentration of the respective compound able to inhibit the growth of microorganisms and these values were determined against previously mentioned isolates bacterial strains based on a micro-well dilution method. The 96-well plates were prepared by dispensing 95 µL of nutrient broth and 5 µL of the microorganism suspensions into each well. 100 µL of the previous prepared highest concentration solution of EO was added into the first wells. Then, 100 µL of their serial dilutions was transferred into eleven consecutive wells. The last well containing 100 µL of DMSO:nutrient broth (1:50), being used as negative control. Consequently, the final volume in each well was 200 µL. Ciprofloxacin was used as a positive control. The plates were incubated at 37 °C for 18–24 h with a stirring of 200 rpm. Microbial growth in each medium was determined by measure of absorbance (Abs) at 600 nm using the ELx 800 universal microplate reader (Biotek Instrument Inc, Highland Park, Vermont, USA). The studied essential oils were tested three times against each organism.

6. Results and discussion

6.1 Yield of essential oils extraction

The yield of EOs extraction by HD and MW-HD are presented in Table 9. In observing these data, what is immediately denoted is that the yield of the oils obtained from the 2016 harvest byproducts is about four times greater than the yield of the oils obtained from the 2017 harvest byproducts. Probably this higher yield reflects only the byproduct variability. The 2016 harvest byproducts comprised thinner and more leafy branches that allowed to extract higher amount of EO, since is it more abundant in leaves. The EOs yields obtained from *Satureja montana* byproducts (Table 9) is about fifty times less than that obtained from the aerial parts of the plant (Table 7). However, the *Satureja montana* byproducts represent 50% of the total plant mass, which means that *Satureja montana* byproducts contains about 2% of all *Satureja montana* essential oil, assuming that all the EO is extracted. Obtaining essential oils from branches promotes a use of material that until now was totally discarded.

Table 9 –Essential oil yield and time process of different extraction methodologies applied.

	Hydrodistillation		**MW-Assisted hydrodistillation**
	2016 Harvest byproducts (n=3)	**2017 Harvest byproducts (n=3)**	**2017 Harvest byproducts (n=3)**
Yield (mg/g)	0.42 ± 0.11	0.14 ± 0.07	0.10 ± 0.01
Time process (min.)	≈120		16±1

By HD and MW-HD similar yields are obtained, although lower standard deviation is denoted by MW-HD. The efficiency of MW-HD is strongly dependent on the dielectric constant of water and the matrix, since heat is originated through the molecular motions within the polar components or ionic species by exposing it to electromagnetic radiation (173). The selective heating of the *in situ* water content of plant material causes tissues to swell and makes the glands and oleiferous receptacles burst and as a consequence the essential oils are released to the environment and evaporated by distillation with the water present (174). The amount of water that is stored intrinsically in the initial dried byproducts (in hydratation process) is a determining factor for the final yield of essential oil extraction. The percentage of hydratation water of the *Satureja montana* byproducts was 55.0±0.4 % (w/w) and this low amount may have limited the yields obtained. If there was a small amount of water inside the

plant cells, a smaller number of water molecules were stimulated to rotate under microwave irradiation, a lower increase of pressure and temperature was generated inside the cells, a smaller number of cells were broken and, as a consequence, part of the target molecules may not have been released into the extracellular medium, not being carried evaporated and recovered in this process (see **Erro! A origem da referência não foi encontrada.** in Appendix) (189). The low standard derivation of percentage of water stored intrinsically in the samples also contributing to a greater reproducibility of MW-HD compared to HD.

Another element that may have limited yields in MW-HD was non-homogeneity of radiation incidence in the sample during the process. Thus, EO extraction may have been more efficient in places where irradiation was more prevalent and inefficient in places where irradiation did not reach. In the pilot scale, this factor has already been overcome by introducing rotation in the drum, which guarantees a homogeneous distribution of the irradiation (174).

The time consumed to recover the essential oils by HD and MW-HD are presented in Table 9. Clearly the MW-HD presents a shorter extraction time, being about 7.5 times smaller than the time required in HD. MW-HD cause the glandular walls to crumble or rupture more rapidly and more efficiently than HD (175) and this happens because MW-HD uses three ways of heat transfer within the sample (irradiation, conduction and convection) and HD uses conduction and convection only. Thus, a reduction of the extraction time would already be expected in MW-HD extraction. The MW-HD extraction time (16 min) in this study (without addition of water in extraction process) is well below the MW-HD extraction time (90 min) of *Satureja montana* aerial parts (water/plant ratio of 20:1 (w/w) (173). The lower timer extraction of MW-HD probably is reflected in lower spent process energy, being this a great advantage for the application of this methodology at industrial level. However, energy studies were no included in this work.

6.2 Chemical characterization of *S. montana* byproducts essential oils

The essential oils of *S. montana* byproducts were subjected to detailed GC-MS analysis in order to determine the impact of the extraction methodology on their volatile constituents, and later to establish a relationship between the chemical composition and the bioactivity of this EOs. Table 10 summarizes the results of chemical composition of both EOs. The volatile compounds found in EOs were grouped by chemical class. The major compound was the monoterpenoid carvacrol. As the internal standard used was 2-undecanol, aliphatic alcohol, the internal standard response factor was calculated relative to Eugenol, which is a monoterpenoid structurally similar to carvacrol. The response factor calculated (1.2033,

R^2=0.9802) was applied to quantify the monoterpenoids, all other groups of compounds being quantified with the application of response factor of 1.0.

Table 10 – Volatile components identified in essential oils obtained from *Satureja montana* byproducts by hydrodistillation (HD) and microwave assisted hydrodistillation (MW-HD), grouped by chemical class.

					HD		MW-HD
No.	**RI_{cal}[d]**	**RI_{lit}[e]**	**Compound**	**Reliability of ID[a]**	**% (w/w) Harvest 2016 (n=3)[b]**	**% (w/w) Harvest 2017 (n=3)[b]**	**% (w/w) Harvest 2017 (n=3)[b]**
			Monoterpenoids				
			Alcohol-type				
1	1428	1526(190)	Linalool	A, B, C	0.5 ± 0.1	0.3 ± 0.1	0.6 ± 0.4
2	1470	1576(190)	Terpineol-4	B, C	0.7 ± 0.0	0.4 ± 0.1	0.4 ± 0.2
3	1528	1668(190)	Borneol	A, B, C	0.5 ± 0.0	0.8 ± 0.3	0.9 ± 0.4
4	1554	1664(190)	α-Terpineol	A, B, C	0.6 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
7	1911	1814(191)	*p*-Cymen-α-ol	B, C	Tr[c]	Tr[c]	Tr[c]
10	1964	2179(192)	Eugenol	A, B, C	Tr[c]	Tr[c]	Tr[c]
11	1969	-	Thymol isomer	C	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
12	1978	1941(190)	Thymol	B, C	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.1
13	1987	1956(190)	Carvacrol	B, C	94.6 ± 2.4	86.6 ± 17.8	81.5 ± 21.3
			Ester-type				
5	1712	-	Carvacrylacetate	B, C	0.2 ± 0.1	0.9 ± 0.7	0.4 ± 0.1
			Oxygenated sesquiterpenoids				
			Oxide-type				
6	1807	1883(190)	Caryophyllene oxide	B, C	0.3 ± 0.1	0.8 ± 0.2	0.5 ± 0.2
			Alcohol-type				
8	1895	1918(190)	Viridiflorol	B, C	Tr[c]	Tr[c]	Tr[c]
9	1941	1933(190)	Spathulenol	B, C	Tr[c]	Tr[c]	Tr[c]
			Identified (%):		97.9 ± 2.0	90.4 ± 18.9	84.9 ± 22.8
			Oil yield (mg/g):		0.42 ± 0.11	0.14 ± 0.07	0.10 ± 0.01

[a]The reliability of the identification or structural proposal is indicated by the following: A, mass spectrum and retention time consistent with those of an authentic standard; B, structural proposals are given on the basis of mass spectral data (*Wiley 275* Library); C, mass spectrum consistent with spectra found in the literature. [b]Estimated concentrations for all compounds were made by peak area comparisons to the

area of a known amount of internal standard (2-undecanol). [c]Tr, traces. [d]Retention indices relative to C8–C22 *n*-alkanes serie. [e]Retention indices reported in the literature for DB-FFAP columns or equivalent.

In both extraction methodologies a similar chemical composition was obtained within a qualitative and quantitative perspective. A total of 13 compounds were identified in EO obtained by HD and MW-HD, accounting for 90.4 - 97.9 % and 84.9% of the total oil content, respectively. Quantitatively, monoterpenoids account for about 84.4 – 97.6% of the EOs and sequiterpenoids represent 0.3 – 0.5%. All monoterpenoids and sesquiterpenoids identified were oxygenated compounds, being this more odoriferous than monoterpene hydrocarbons (174). If there are only oxygenated compounds, this explains the lack of quantitative differences between the different extraction methodologies. The predominance of oxygenated monoterpenes over hydrocarbons monoterpenes as reported by Wang et al. (155) would not therefore be visible in these samples.

Byproducts *S. montana* L. EO are characterized by a high content of the phenolic monoterpenoid carvacrol (≈82 - 95%). This high level of carvacrol in *S. montana* L. byproducts EO is in accordance with the value reported by Kustrak *et al*. (171), which until now was the highest value detected in aerial *S.* montana L. EO (84%). As Friedman et al. (193) reviews, Carvacrol is synthesized via the mevalonate pathway, where the key intermediate, mevalonic acid, is formed, which is later transformed into *γ*-terpinene and then undergoes aromatization to *p*-cymene and hydroxylation to carvacrol and thymol (see **Erro! A origem da referência não foi encontrada.** in Appendix). There is clear propensity in the formation of carvacrol in relation to the others isomers (such thymol and thymol isomer) in the hydroxylation step, as can be seen by estimated concentration of this isomers in Table 10, maybe due to their natural origin, environmental, seasonal and genetic factors (156,158,159,171). The carvacrol concentration was similar in the 2016 and 2017 harvest and in both extraction methodologies, although small variations can be denoted.

Leaves and flowers of *Satureja montana* have terpenoids in form of glycoconjugates, including carvacrol, although in small concentration (158). In *Satureja montana* byproducts there are still no studies about it but the extent to which the terpenoids are free or in conjugated form will therefore be a factor that will influence the yield of essential oil extraction. In the future, it would be interesting to evaluate if a previous enzymatic treatment would have any impact on the EO extraction yield.

6.3 Chemical characterization of overplus hydration water extract (OHWE)

The overplus hydration water resulted from the hydration process of the *Satureja montana* branches before the MW-HD. This water was analyzed in the sense of trying to find

out if part of the EO compounds were released in the hydration process, thus discarded to the process of extraction by MW-HD.

After the hydration process of about 100 g of branches, about 860 mL of overplus hydration water is recovered. Two replicates of OHWE were obtained in a yield of 0.16 and 0.17 mg/ g of branches, respectively. After filtration, the volatile components were identified and chemically grouped as shown in

Table 11. A total of 27 compounds were identified in OHWE, 9 of which are monoterpenoids and 1 sesquiterpenoid. With the exception of dihydroactinidiolide, all other free terpenenoid found in OHWE are compounds found in the byproducts *Satureja montana* EOs. OHWR contains about 10.1% free terpenoids, whereas *S. montana* EO obtained by MW-HD contains about 84.9%. Quantitatively, for each extraction the entire OHWE contains about 5 times less free terpenes than *S. montana* EO. This means that effectively a portion of the compounds were removed in the hydration process and did not enter in the extractive process of the essential oils by MW-HD. In the specific case of carvacrol and assuming that all carvacrol has been extracted from the plant, it can be said that about 15% of this compound is removed by diffusion in the hydration process. In the background, the hydration process of the branches was simultaneously a slow extractive process at room temperature and for long hours, where many other compounds are extracted in addition to free terpenes. Among these compounds are aliphatic carboxylic acids, aromatic compounds and others. All identified compounds only represent 22.3 – 27.9% of OHWE. Among the unidentified compounds may be larger compounds that have been retained in the filter or less volatile compounds that could not be identified by GC-MS.

Before the application of the microwave to the extraction of EO from dry samples at industrial level, applications for the overplus hydration water should be addressed in order to promote a circular economy, thus avoiding the creation of a new waste.

Table 11- Volatile components identified in overplus hydration water extract (OHWE), grouped by chemical class.

No.	RI_{cal}[d]	RI_{lit}[e]	Compound	Reliability of ID[c]	% (w/w) OHWR 1[a]	% (w/w) OHWR 2[a]
			Monoterpenoids			
			<u>*Alcohol type*</u>			
1	1421	1526(190)	Linalool	A, B, C	0.06	0.10

2	1465	1576 (190)	4-Terpineol	B, C	0.04	0.05
3	1530	1668 (190)	Borneol	A, B, C	0.07	0.10
4	1548	1664(190)	α-terpineol	A, B, C	0.13	0.10
8	1903	1814(191)	*p*-Cymen-8-ol	B, C	0.03	0.08
9	1959	2179(192)	Eugenol	A, B, C	0.07	0.09
10	1969	-	Thymol isomer	C	Tr[b]	Tr[b]
11	1973	1941 (190)	Thymol	B, C	0.20	0.03
12	1983	1956 (190)	Carvacrol	B, C	7.87	10.32
			Ester-type			
17	2256	-	dihydroactinidiolide	B	0.32	0.31
			Sesquiterpenoids			
			Oxide-type			
5	1803	1883 (190)	Caryophyllene oxide	B, C	0.07	0.11
			Aliphatic carboxylic acids			
7	1879	2024(194)	Caprylic acid	B	0.16	0.13
15	2082	2276(195)	Capric acid	B	0.17	0.19
18	2241	2471(195)	Lauric acid	B	0.04	0.05
22	2561	2871(196)	Palmitic acid	B	0.51	0.53
24	>2494	-	stearic acid	B	0.17	0.38
26	>2494	-	Linoleic acid	B	0.18	0.30
27	>2494	-	7,10,13-hexadecatrienoic acid	B	0.27	0.29
			Aromatic compounds			
14	2076	2273(195)	2,6-Dimethoxyphenol	B	0.06	0.09
19	2287	2571(195)	Vanillin	A, B	1.48	1.51
21	2337	-	acetovanillone	B	0.24	0.35
23	2494	-	syringaldehyde	B	0.74	0.84
25	>2494	-	3-(*p*-hydroxy-m-methoxyphenyl)-2-	B	0.29	0.38

			propenal			
			Others			
6	1822	-	trans-β-ionone-5,6-epoxide	B	0.03	0.04
13	2026		Methylethylmaleimide	B	0.07	0.05
16	2099	-	Dihydromethyljasmonate	B	0.04	0.04
20	2321	-	2,6-dimethyl-3-(methoxymethyl)-p-benzoquinone	B	0.11	0.13

[a]Estimated concentrations for all compounds were made by peak area comparisons to the area of a known amount of internal standard (2-undecanol). [b]Tr, Traces. [c]The reliability of the identification or structural proposal is indicated by the following: A, mass spectrum and retention time consistent with those of an authentic standard; B, structural proposals are given on the basis of mass spectral data (*Wiley 275* Library); C, mass spectrum consistent with spectra found in the literature. [d]Retention indices relative to C8–C22 *n*-alkanes. [e]Retention indices reported in the literature for DB-FFAP columns or equivalent.

6.4 Antimicrobial activity of *S. montana* essential oil

The antimicrobial activities of *S. montana* essential oil were qualified by inhibition zone diameter using the agar disc diffusion method and quantified by determining the minimal inhibition concentration (MIC) using a broth microdilution assay against Gram-positive and Gram-negative bacteria. *S. montana* EOs obtained by HD and MW-HD were mixed and their antimicrobial potential was evaluated as a single sample, since they presented an identical chemical composition, qualitatively and quantitatively, as described above. The results of the disc diffusion testing are listed in Table 12 (assay 1) and in Figure 10 (assay 2). The DMSO or sterile water negative control showed no inhibition effects.

Two different inhibition zones are visible in disc diffusion testing plate (Table 12), total and partial inhibition, as can be seen in Table 12. The formation of these growth inhibition zones around *S. montana* EO disc for all tested bacterial cultures showed their antimicrobial activity. It is known that the hydrophobicity of EOs components enables them to partition in the lipids of the bacterial cell membrane, disturbing the structures and rendering them more permeable, being this loss of differential permeability considered the cause of cell death of microorganisms (162). In specific case of tested EO, the antibacterial activity is probably due to the presence of carvacrol, not only because of its high abundance in *S. montana* EO tested, but also because of its high specific activity as compared to other EO components, being considered one of the main components of certain EOs that exerts antimicrobial activity

(193,197). In previous studies, there appears to be a relationship (even if not linear) between the amount of carvacrol from different *S. montana* EOs and their antimicrobial effect: EOs with high carvacrol content showed a great antimicrobial potential (167) while EOs with a lower carvacrol content showed a less antimicrobial potential (156). To date, the EO under study exhibits the highest inhibition diameters against the same microorganism's species (156,167), perhaps because it is *S. montana* EO with a higher percentage of carvacrol whose antimicrobial activity was tested

The antimicrobial activity of the carvacrol is highly related to some structural requirements such as the presence of aliphatic ring substituents and hydroxyl group (197) which make it more active than other monoterpenoids. The synergism of this compound with the other minor terpenoids present in EO tested is a hypothesis that should not be excluded (154,198). Pei *et al*. (198) verified that carvacrol/thymol and carvacrol/eugenol have a synergic effect and this may be indicative of that synergistic effects in *S. montana* EO may also occur, since this compounds are present in simultaneous. The thymol isomer can also have synergistic effects, due to its structural similarities with thymol; however, to date there are no studies evaluating synergistic effects with this isomer.

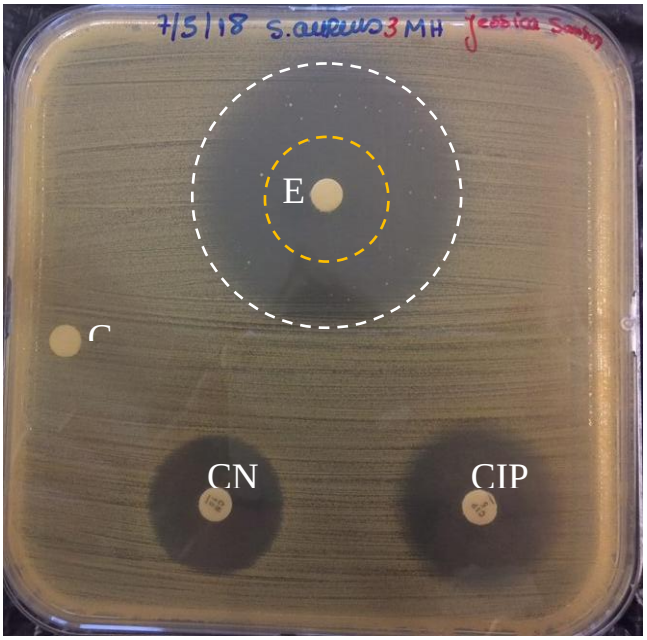

Figure 9 – Plate with Inhibition zones of *S. aureus* ATCC 6538 against negative control (C, 10µL DMSO), gentamicin (CN, 10 µg) and ciprofloxacin (CIP, 5 µg) and *Satureja montana* essencial oil (EO, 0.5 µL) using agar disc diffusion method. The yellow flashing line represents the zone of total inhibition, where no colony was detected, and the blinking white line represents the zone of partial inhibition, where some colonies are visualized, but where there is still clear inhibition.

The inhibition zones (Table 12) reveal different susceptibility of different microorganisms to the *S. montana* EO. The amount of disc-tested oil for *S. aureus* ATCC 6538

(Gram-positive bacteria) in Assay 1 was 0.5 µL, four times lower than the volume tested against the remaining strains (Gram-negative bacteria), yet had an inhibition diameter equal to or greater than the diameters generated in the remaining strains. *Salmonella enterica* sv Anatum SF2 and *E. coli* ATCC 25922 strains (Gram-negative bacteria) were therefore much less susceptible than *S. aureus* ATCC 6538 (Gram-positive bacteria) to EO. These results are in agreement with the results obtained by Chao *et al*. (199) that investigated the effects of 45 EOs, among which S*atureja montana* EO (obtained from aerial parts of plant), on a broad spectrum of microorganisms, including eight different genera of bacteria, four of which were Gram-positive and four of which were Gram-negative. The relative tolerance of Gram-negative bacteria to essential oils is due to the presence of hydrophilic lipopolysaccharides (LPS) in outer membrane which create a barrier toward macromolecules and hydrophobic compounds, blocking the penetration of these components though the target cell membrane (160). Gram-positive bacteria, in turn, possess a permeable cell wall that usually does not restrict the penetration of antimicrobials, since the major component cell wall is peptidoglycan covalently linked to teichoic and teichuronic acids (200).

Table 12 - Zones of growth inhibition (mm) of byproduct *S. montana* essential oil using agar disc diffusion method (Assay 1)

Microorganisms	***S. montana* L. EO (n=3)**	**Antibiotic CIP[a]**	**Antibiotic Gen[b]**	**Sterile water**
Total inhibition				
E. coli ATCC 25922	25 ± 3[c]	34 ± 1	21 ± 0	ND[e]
Salmonella enterica sv Anatum SF2	13 ± 1[c]	31 ± 1	20 ± 1	ND[e]
S. *aureus* ATCC 6538	23 ± 4[d]	28 ± 1	25 ± 1	ND[e]
Partial inhibition				
E. coli ATCC 25922	33 ± 2[c]	42 ± 1	27 ± 0	ND[e]
Salmonella enterica sv Anatum SF2	20 ± 4[c]	38 ± 1	25 ± 0	ND[e]
S. aureus ATCC 6538	40 ± 8[d]	NPI[f]	NPI[f]	ND[e]

[a]CIP, ciprofloxacin (5 µg/disc). [b]Gen, gentamicin (10 µg/disc). [c] inhibition zone in diameter around the discs impregnated with 1.75 mg of *S. montana* EO (containing approximately 1.53 mg of carvacrol). [d] inhibition zone in diameter around the discs impregnated with 0.44 mg of *S. montana* EO (containing approximately 0.38 mg of carvacrol). Inhibition zones values include

the disk diameter (6.0 mm). [e]ND, no inhibitory effect was detected. [f]NPI, no partial inhibition was detected beyond the total inhibition diameter.

The total inhibition zones diameter was shown to have a linear relationship to the amount of *S. montana* EO impregnated in each test disc for each microorganism (Figure 10), with correlation coefficients (R^2) of 0.9543, 0.9552, and 0.8258 for *S. aureus*, *E. coli* and *Salmonella,* respectively. The susceptibility of each strain seems to be reflected in a higher value of slope, since the largest slope corresponded to *S. aureus*, followed by *E. coli* and *Salmonella*, with slopes more comparable to each other.

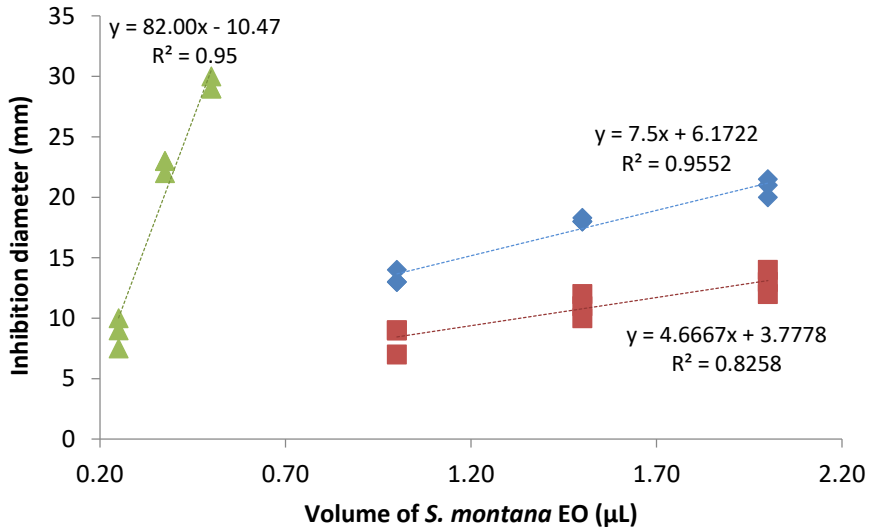

Figure 10 - Relation between the amount of *Satureja montana* essential oil and total inhibition zones (mm) against *E. coli* ATCC 25922 (◆), *Salmonella enterica* sv Anatum SF2 (▲) e S. *aureus* ATCC 6538 (▲) using agar disc diffusion method. Each *S. montana* EO volume tested was done in triplicate. The sterile water (E. *coli* ATCC 25922 and *Salmonella enterica sv* Anatum SF2) or DMSO negative control (*S. aureus* ATCC 6538) showed no inhibition effect. The inhibition diameters of ciprofloxacin positive control was 31±1, 34±0 and 26±0 for each microorganism, respectively, and the inhibition diameter of gentamicin positive control was 20±1, 20±0 and 24±0.

The MIC value is cited by most researchers as a measure of the antibacterial performance of EOs (154). EO MIC for different tested strains were listed in Table 13. These values also reflect the susceptibility of each strain to EO: *S. aureus* presents a lower MIC value, followed by *Salmonella enterica* and *E. coli*. The obtained values are below the values reported for the same bacterial species, 780 µL/mL for *Staphylococcus aureus* ATCC 25923, 780 µL/mL for *Escherichia coli* ATCC 35218, and 390-780 for *Salmonella* spp. strains (167). The carvacrol MICs for the same tested strains were also represented in Table 13. These values are above the MIC values of the tested EO for the corresponding strains, although they are very close values Table 13. This may mean that effectively the antimicrobial activity of the tested EO is largely due to carvacrol.

The calculated MIC values are theoretical values that reflect only the antimicrobial activity tested *in vitro* and do not effectively represent the concentration to be added in animal feed. The *in vivo* activity will be dependent on other factors such as the interaction between the food matrix and the EO components and its bioavailability. However, this type of antimicrobial test is necessary for first screening of EO activity.

Table 13 – Minimal inhibition concentration (MIC) of byproducts *S. montana* essential oil. The values are expressed as µg/mL of liquid medium

Microorganisms	**S. *montana* L. EO_{cal}[a]**	**Carvacrol MIC_{lit}[b]**
E. coli ATCC 25922	225	250(201)
Salmonella enterica sv Anatum SF2	250	NR[c]
S. *aureus* ATCC 6538	150	175(202)

[a] The negative control with 1% of DMSO showed no inhibition effects. [b]The values presented were not obtained in this study. [c]NR, no reported value for respective strain

Other EOs with high levels of carvacrol have already been tested *in vivo* and are active as alternatives to growth antibiotic promoters, Thyme's EOs being an example. The supplementation of 60 mg thyme essential oil (67.8% carvacrol, 4.9% γ-terpineol, 4.7% cymene, 3.4% thymol and 3.2% linalool) by kg of chickens` diet resulted in significantly higher body weight gains and better-feed efficiency as compared to that of control group, probably due to a positive effect generated on the intestinal microflora (203). If this *S. montana* EOs tested in this study have a higher concentration of carvacrol (the main active compound), a lower dosage may be required in animal feed to indicate positive effects on animal health and its growth. In a rough approximation and without accounting for any synergistic or antagonistic effects, incorporation of approximately 46 mg of S. montana EO per kg of diet would produce biological effects equivalents to those obtained by Denli *et al*. (203).

6.5 Carbohydrate composition of skin and top onion

Onion skin dry weight was composed by 49% of carbohydrates (Table 14), namely glucose (47% mol) and uronic acids (44%). Top onion has lower carbohydrate content (30%), being also mainly constituted by glucose (46%) and uronic acids (32%). These results were according with Jaimes *et al*. (16) that has already studied carbohydrate composition of onion tissues of three varieties, revealing that cellulose and pectic polysaccharides are the major components of all tissues. Within the uncharacterized fraction, protein, lipids, sulfur-

containing compounds, flavonoids and other phenolic compounds may be present, as described in more detail in section 3.3).

6.6 Sequential extraction and structural analysis of skin and top onion sugars

The sequential extraction of skin and top onion carbohydrates was performed with an initial extraction with ethanol 80% (v/v), the obtained ethanol insoluble residue (REt) comprising cell walls, was submitted to a hot water extraction. The purpose of this sequential extraction from onion byproducts was to obtain and study the presence of possible compounds with prebiotic potential, such as fructooligosaccharides, compounds already described in the onion bulb. The mass yields and sugars composition of the extracts and the final residues of skin and top onion extractions are shown in Table 14 (total sugars) and Table 15 (free sugars) and the glycosidic linkages composition are shown in Table 16.

Table 14 – Extraction yields and carbohydrate composition of the fractions obtained by sequential extraction of skin and top onion powder

	η %	%mol								Total sugars
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	µg / mg
Skin onion		Tr[c]	Tr[c]	**1**	**4**	**2**	**2**	**47**	**44**	**493**
SnEt	7[a]	1	1	1	Tr[c]	2	Tr[c]	91	5	291
REt	86[a]	1	Tr[c]	1	3	1	2	48	43	540
SnAq	2[b]	2	Tr[c]	5	2	6	10	21	53	334
Residue	76[b]	1	Tr[c]	1	4	1	2	40	51	646
Top onion		**1**	**1**	**4**	**7**	**4**	**5**	**46**	**32**	**301**
SnEt	4[a]	1	1	20	3	3	1	56	15	153
REt	77[a]	1	Tr[c]	3	6	3	3	45	38	451
SnAq	14[b]	4	1	8	4	5	11	21	47	224
Residue	78[b]	Tr[c]	0	1	5	2	2	42	47	546

[a]The values correspond to yields calculated in initial sample mass basis. [b] The values correspond to yields calculated in REt mass basis. [c]Tr, traces.

The initial extraction with ethanol 80% (v/v) from onion skin powder solubilized 7% of initial material, counting only 29% of carbohydrate content (Table 14) of which 13% were free sugars (Table 15). This means that many other compounds have been solubilized in addition to carbohydrates. Within solubilized sugars are mainly glucose (91%). Glycosidic-linkage analysis of this fraction showed that this glucose was mainly as terminally linked-Glc (Table 16). Taking into account that the onion contains several flavonoids and other glycosylated phenolic (see Table 4 in section 3.3.4), this large amount terminally linked-Glc may indicate the presence of

this type of compounds in this extract. The free sugars solubilized in ethanol were mainly glucose (75%) and fructose (19%) (Table 15).

From top onion, similar results were obtained after ethanol extration, 4% of initial material were solubilized in ethanol 80% (v/v), counting for 15% of carbohydrates where 8% were free sugars. Glucose is main constituent (56%) of present carbohydrates, however in a less amount than skin onion (Table 15). This glucose was also mainly in the form of terminally linked-Glc. In addition to glycosylated phenolic compounds, the arabinose (20%) and uronic acids (15%) probably indicate the presence of arabinan side chains associated with pectic polysaccharides (Table 16).

Table 15 - Extraction yields and free sugar composition of the fractions obtained by sequential extraction of skin and top onion powder

	η %[a]	%mol										Total free sugars
		Rha	Fuc	Ara	Xyl	myo innositol	Fru[b]	Glc	Gal	Suc[c]	Tre[d]	µg / mg
Skin onion		**1**	**4**	**14**	**3**		**5**	**65**	**3**			**25**
SnEt	7	-	Tr[f]	2	Tr[f]	1	19	75	1	1	1	129
SnAq	2	-	Tr[f]	2	Tr[f]	9	17	66	2	1	3	45
Top onion		**2**	**4**	**37**	**8**		**6**	**31**	**6**			**20**
SnEt	4	-	1	33	6	3	23	25	1	Tr[f]	8	82
SnAq	14	-	Tr[f]	26	4	6	20	34	2	Tr[f]	6	38

[a]The values correspond to yields calculated in REt mass basis. [b]The fructose content was calculated based on the peaks of acetylated mannitol and acetylated glucitol, assuming that all detected acetylated mannitol results from derivatization of fructose. [c]suc, sucrose. [d]tre, trehalose. [f]Tr, traces.

Hot water extraction from skin and top onion REt led to extraction of 2 and 14% of the material, respectively, accounting for 33 and 22% of carbohydrate content (Table 14) from those 5 and 4% of free sugars, respectively (Table 15). In both extractions, the extracted carbohydrate is composed mainly of uronic acids (53 and 47%, respectively) and this shows that pectic polysaccharides were extracted. The presence of galactose in a percentage of 10 and 11% skin and top onion, respectively, appears to indicate that the extracted pectic polysaccharides are mostly homogalacturonans substituted with galactans, polysaccharides already described in onion tissues (16,204). Glycosidic linkages analysis revealed that in addition to homogalacturonans, xylogucans fragments could be extracted with hot water once terminally- and (1→2)-linked-xylose, (1→4)- and (1→4,6)-linked glucose were found (205). Methylation analyses still revealed that (1→4)-galactans chains (206), (1→5)-arabinans chains (206) and arabinogalactans chains type I and type II were diagnostic linkages (terminally-

,(1→3)-, (1→4)-, (1→3,6)-, (1→6)-linked galactose and terminally- and (1→5)- linked-arabinose were found (207)) can be present in hot water extracts. The obtained residues contained a percentage of carbohydrates of 65 or 55% such as cellulose, and less soluble pectic polysaccharides.

All obtained extracts (SnEts and SnAqs) obtained from the skin onion and top onion, respectively) exhibit only small fractions of disaccharides (sucrose and trehalose). Trehalose is a disaccharide already described as prebiotic (208), however, the amounts detected in the all fractions are so low that they would probably not be sufficient to present activity. No trisacharides were detected which means that if fructooligosaccharides were present in these fractions, they would have a degree of polymerisation higher than 3. Glycosidic-linkage analysis revealed that these fractions contains a small amount of (1→2)-linked fructose and traces of terminally-linked fructose, which means that may be present fructooligosaccharides with high polymerization degree or even inulin. The presence of free fructose, glucose and sucrose may be the result of degradation of fructans, thus justifying the low amount of these molecules.

Table 16 – Glycosyl linkage composition (mol%) of the fractions obtain from skin and top onion by sequential extraction

Glycosidic linkage	Skin onion		Top onion	
	SnEt	**SnAq**	**SnEt**	**SnAq**
T-Fuc	-	1	-	1
Total	**-**	**1**	**-**	**1**
T-Ara	-	1	Tr[a]	2
2-Ara*f*	-	Tr[a]	-	1
5-Ara*f* / 4-Ara*p*[b]	Tr[a]	2	Tr[a]	2
3- Ara*p* /3-Ara*f*[b]	-	1	-	1
2,5-Ara*f* / 2,4-Ara*p*[b]	-	Tr[a]	-	1
Total	**-**	**4**	**-**	**6**
T-Xyl	Tr[a]	2	1	2
4-Xyl/2-Xyl[b]	Tr[a]	3	1	4
Total	**-**	**4**	**2**	**6**
T-Man	1	4	2	4
6-Man	3	2	4	3
Total	**4**	**3**	**6**	**7**
T-Fru	Tr[a]	Tr[a]	Tr[a]	Tr[a]
2-Fru	1	3	3	4

Total	**1**	**3**	**3**	**4**
T-Glc	93	44	83	36
2-Glc	-	2	-	Tr[a]
3- Glc	1	1	1	3
4- Glc	1	12	3	9
6- Glc	-	1	Tr[a]	2
2,6-Glc	-	Tr[a]	2	4
3,6- Glc	-	1	-	2
4,6-Glc	Tr[a]	2	-	2
Total	**95**	**63**	**88**	**57**
T-Gal	Tr[a]	3	1	3
4-Gal	-	6	Tr[a]	2
3-Gal	Tr[a]	3	Tr[a]	6
6-Gal	-	3	Tr[a]	2
3,6-Gal	-	5	-	5
Total	**-**	**19**	**1**	**18**

The calculated molar percentages do not encompass oligosaccharides or polysaccharides containing uronic acids. [a]Tr, traces. [b]Glycosidic linkages not distinguishable by the mass spectrum and retention time

6.7 Influence of microwave assisted extraction on yield and sugar composition of skin onion powder

The microwave superheated water extraction was applied to onion skin powder using different temperatures (120, 140 or 160 °C) and extraction solvents (distilled water or ethanol 65%). These extractions were done in order to determine the yield and composition of recovered carbohydrate when microwave assisted extraction is used. The purpose was to obtain possible compounds with prebiotic potential, applying higher temperatures / pressures in order to increase the yields. The mass yields and sugars composition of the extracts and final residues of onion skin microwave assisted extractions are shown in Table 17 (total sugars) and Table 18 (free sugars).

The amount of material extracted with ethanol 65 % (v/v), MW Et65, on onion skin dry basis, did not change (13 – 14 %) with the increase of extraction temperature (Table 17), however, the carbohydrate content of extracted material increased from 26% to 35%. The composition of the carbohydrates extracted was altered with the rise in extraction temperature: the glucose content decreased (from 78 to 45%) and the content of uronic acids increased (from 10 to 31%). The increase of uronic acids was also with an observed increase of rhamnose (from 4 to 10 %), arabinose (from 1 to 4 %) and galactose (from 2 to 6 %). This

means that at higher temperatures more pectic polysaccharides were extracted, being in agreement with Bagherian *et al*. (209).

Table 17 - Effect of temperature and solvent on extraction yields and sugar composition of the fractions obtained from onion skin by microwave superheated water extraction.

Fraction	Temp. (°C)	η (%)	%mol								Total sugar
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	ug/mg
MW Et65	120	13	4	1	1	1	2	2	78	10	262
	140	13	5	1	1	1	2	3	65	22	271
	160	14	10	1	4	1	2	6	45	31	346
MW Et65 residue	120	76	2	Tr[a]	1	3	2	2	45	44	791
	140	75	2	Tr[a]	1	3	1	2	37	53	830
	160	73	3	Tr[a]	Tr[a]	3	2	2	47	43	873
MW H_2O	120	7	2	1	3	1	3	5	62	24	390
	140	10	3	1	4	1	3	6	51	31	446
	160	18	7	Tr[a]	4	4	5	9	26	45	414
MW H_2O residue	120	78	1	Tr[a]	1	3	2	2	44	47	746
	140	83	1	Tr[a]	1	3	2	2	42	50	790
	160	66	1	Tr[a]	Tr[a]	3	2	1	59	34	830

The yield values were calculated in initial samples mass basis.

Contrary to the observed in microwave ethanol extractions, the yields of water extracts (MW H_2O) increased from 7 to 18 when the extraction temperature increased from 120 to 160 ° C (Table 17). The carbohydrate content in these fractions is generally higher (39 - 45 %) than in the extracts obtained with ethanol. The compositional changes of total carbohydrates in water extracts were similar to those denoted in ethanol extracts.

Free sugars content decreased in ethanol and water extractions with the rise of extraction temperature (from 16 to 11% and from 23 to 12%), as can be seen in Table 18, which may have occurred due to a greater thermal degradation of the free monosaccharides. Glucose and fructose are the main free sugars present in all extracts, however, there is a higher concentration of the other neutral monosacharides (arabinose, fucose, xylose and galactose) at 160 ° C, which means that at this temperature some depolymerization of the side chains of pectic polysaccharides and xyloglucans may have occurred. Very small amounts of the disaccharides sucrose, trehalose and kojibiose were detected and no trisaccharides were

detected. The imposed conditions on the various microwave extractions did not, therefore, provide the formation of small oligosaccharides to DP 3 by breaking inulin polymers. However, further analysis will be required to assess whether oligosaccharides with DP greater or equal than 4 were formed.

Table 18 - Effect of temperature and solvent on extraction yields and free sugar composition of the fractions obtained from onion skin by microwave superheated water extraction.

Fraction	Temp. (°C)	η (%)	%mol												Total free sugar
			Rha	Fuc	Ara	Xyl	Fru[a]	Gal	Glc	Di1[b]	Di2[b]	Suc[c]	Tre[d]	Koj[e]	ug/mg
MW Et65	120	13	Tr[f]	Tr[f]	2	Tr[f]	11	Tr[f]	86	-	-	Tr[f]	1	Tr[f]	156
	140	13	Tr[f]	Tr[f]	2	Tr[f]	9	Tr[f]	87	-	-	Tr[f]	1	Tr[f]	139
	160	14	1	1	5	1	13	Tr[f]	78	-	-	1	1	Tr[f]	114
MW H_2O	120	7	Tr[f]	Tr[f]	2	Tr[f]	11	Tr[f]	84	-	-	Tr[f]	1	Tr[f]	232
	140	10	Tr[f]	1	3	Tr[f]	11	1	82	-	-	Tr[f]	1	Tr[f]	182
	160	18	9	6	13	7	10	16	38	Tr[f]	Tr[f]	Tr[f]	Tr[f]	Tr[f]	117

The yield values correspond to yields calculated in initial samples mass basis. [a]The fructose content was calculated based on the peaks of acetylated mannitol and acetylated glucitol, assuming that all detected acetylated mannitol results from derivatization of fructose. [b]Di, disacharide not identified. [c]Suc, sucrose. [d]Tre, trehalose. [e]Koj, Kojibiose. [f]Tr, traces.

Taking into account the amount of pectic polysaccharides contained in skin onion, it will be interesting to investigate whether pectic oligosaccharides were produced in the various applied microwave extractions, since these molecules have been identified as emerging prebiotics (64). In the future, it would also be interesting to apply enzymatic or acid treatments to onion byproducts and evaluated the structure and bioactivity of produced oligosacharides.

7. Concluding remarks

Carbohydrate composition analysis of onion byproducts revealed that they are poor in fructans, contrary to bulb onion, but rich in pectic polysaccharides. In order to value these byproducts, pectic oligosacharides can be obtained by enzymatic or acidic treatment and their prebiotic potential should be exploited. More studies will be necessary to evaluate onion byproducts as possible ingredients in animal diet.

Satureja montana byproducts can be harnessed to obtain essential oils. The chemical composition of these essential oils was not influenced by the plant harvest nor by the two extraction methodologies tested (hydrodistillation and microwave assisted hydrodistillation). However, it is to be noted that the essential oils obtained by microwave assisted hydrodistillation are achieved with a shorter process time, contrary to what happens when they are obtained by hydrodistillation. They are constituted mainly by carvacrol, this being the main active compound responsible for its antimicrobial activity.

The present work showed that *S. montana* byproducts essential oils was have antimicrobial activity against Gram-positive and Gram-negative bacteria related with poultry infections. In the future, this antimicrobial potential could be exploited within the livestock sector towards reducing / abolishing growth promoter antibiotics, while promoting a circular economy. *In vivo* assays will still be required to confirm the applicability of these essential oils as animal health promoters. It would still be interesting to study the effect of these phytobiotics together other classes of alternatives to growth promoter antibiotics, such as prebiotics, noting if there is a synergistic or antagonistic effect.

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9. Appendix

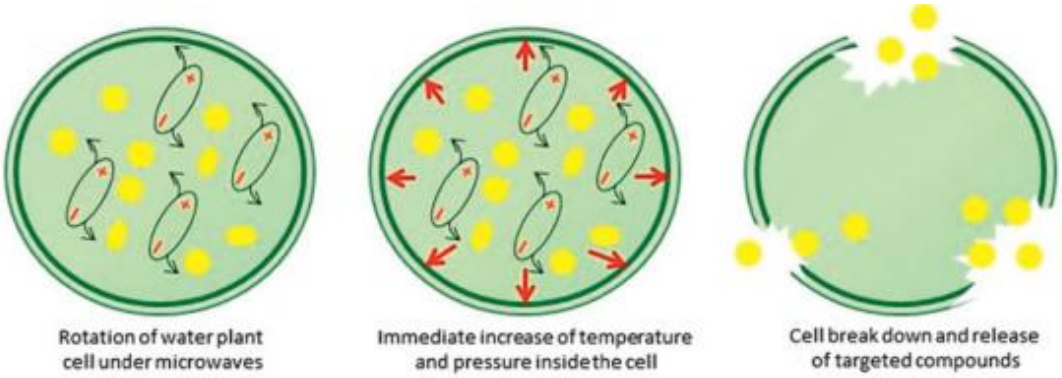

Figure 11- Mechanism of microwave assisted hydrodistillation (reproduced from Chemat *et al*.(189))

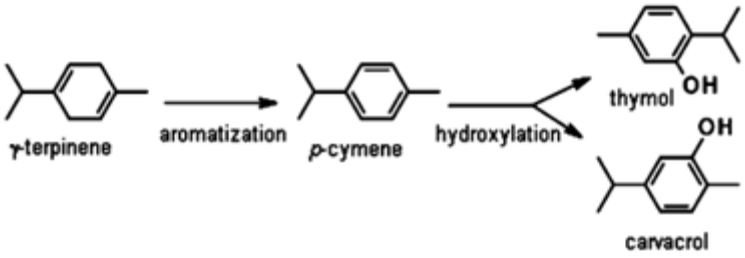

Figure 12 - Biosynthesis of carvacrol or thymol in aromatic plants (adapted from Friedman *et al*. (193))